IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Tracy Ann Willson, et al.	**Examiner:**	Landsman, Robert S.
Serial No.:	09/868,813	**Art Unit:**	1647
Filed:	February 26, 2002	**Docket:**	14730
For:	SOCS-BOX CONTAINING PEPTIDES		

Confirmation No.: 6320

Commissioner for Patents
Alexandria, VA 22313-1450

DECLARATION OF DR. NICOS A. NICOLA UNDER 37 C.F.R. §1.132

Sir:

I, NICOS A. NICOLA, hereby declare as follows:

1. I am one of the co-inventors named in the above-identified application. I am currently Assistant Director and NHMRC Senior Principal Research Fellow at the Walter and Eliza Hall Institute of Medical Research located at 1G Royal Parade, Parkville, Victoria 3050, Australia.

2. I hold a Bachelor of Science with Honours (BSc (Hons)) Degree and a Doctor of Philosophy (PhD) Degree in biochemistry. A true and correct copy of my curriculum vitae is attached hereto as **Exhibit A.**

3. I have reviewed the above-identified application ("the '813 application") and I am familiar with the subject matter disclosed and claimed therein. I understand that the '813 application presently claims a method of identifying an agent which inhibits protein degradation in cells mediated by elongin C based on detection of inhibition of the interaction between a SOCS box and elongin C. The '813 application also claims a method of identifying an agent which promotes protein degradation in cells mediated by elongin C based on detection of an enhanced interaction between a SOCS box and elongin C.

4. I have been asked to comment on whether the '813 application provides adequate guidance for those skilled in the art to practice the claimed methods, particularly in respect to methods of identifying an agent that inhibits or promotes degradation of proteins without a SOCS-box.

5. It is my scientific opinion that the '813 application provides ample guidance with respect to the claimed methods insofar as degradation of proteins without a SOCS-box is concerned. My opinion is based on a review of the disclosure of the '813 application as well as several published articles, which are further discussed below.

6. It is disclosed in the '813 application that SOCS-box-containing proteins form a family of adaptor proteins which terminate cell signaling by targeting critical molecules for intracellular degradation. See , e.g., page 2, line 30 to page 3, line 2 of the '813 application. According to the '813 application, the SOCS box in proteins facilitates the presentation of proteins bound to the SOCS box to the ubiquitination and/or proteasomal compartments via an

interaction with elongin B or elongin C. See page 2, lines 28-29; and page 34, line 19 through page 36, line 4.

7. Clearly, proteins presented by SOCS-box-containing proteins for intracellular degradation bind to SOCS-box-containing proteins, but do not necessarily contain a SOCS-box themselves. In this regard, the '813 application provides examples of proteins that do not contain a SOCS box, but bind to SOCS-box-containing proteins, i.e., JAKs (signaling molecules that bind to SOCS-1 and SOCS-3). See page 10, lines 14-23 of the '813 application. The '813 application also describes how to analyze the interactions among SOCS-box-containing proteins, elongins, and signaling molecules that bind to SOCS-box-containing proteins; and how to determine whether the interactions are direct or indirect. See page 36, line 17 to page 37, line 7, for example.

8. Therefore, it is my scientific opinion that based on the teaching of the '813 application, the skilled artisan would be able to readily identify proteins, including proteins that do not contain a SOCS-box, that interact with a SOCS-box-containing protein for targeted intracellular degradation. Further, those skilled in the art would be able to identify agents that inhibit or enhance the interaction between SOCS-box-containing proteins and elongin B/C, and further determine whether such agents inhibit or promote degradation of proteins without a SOCS-box that bind to a SOCS-box-containing protein.

9. Subsequent to the filing of the present application, several articles have been published that demonstrated intracellular degradation of different signaling molecules, all without a SOCS-box, through interactions with SOCS-box-containing proteins.

10. In an article published in *Molecular and Cellular Biology* 22: 3316-3326 (2002) (**Exhibit B**), Ungureanu and colleagues demonstrated that the turnover of activated Jak2 is regulated by the ubiquitin-dependent proteolysis pathway and is dependent on the presence of the SOCS-box of SOCS-1. See, in particular, page 3320, right column, last paragraph to page 3322, left column, first paragraph; and page 3322, right column, last paragraph to page 3323, left column, first paragraph.

11. In another article published in *J. Biological Chemistry* 276(16): 12530-12538 (2001) (**Exhibit C**), Kamizono and colleagues demonstrated that the activity of the fusion protein, TEL-JAK2, was down-regulated through SOCS-box mediated proteasomal degradation of the fusion protein. The authors also demonstrated that degradation of TEL-JAK2 depended on its high-affinity binding to SOCS-1, and that the SOCS-box interacted with Cullin-2 and promoted ubiquitination of TEL-JAK2. See, in particular, page 12532, right column, first paragraph to page 12534, left column, first paragraph.

12. Similarly, SOCS proteins have been reported to promote ubiquitination and degradation of two additional proteins without a SOCS-box – VAV and FAK. See *J. Biol. Chem.* 275: 14005-14008 (2000) (**Exhibit D**); and *EMBO* 22(19): 5036-5046 (2003) (**Exhibit E**).

13. The articles in the attached **Exhibit A-D** have confirmed the basis of the methods claimed in the '813 application, i.e., SOCS-box-containing proteins act as adaptor proteins which target critical molecules for intracellular degradation, including degradation of molecules that do not contain a SOCS-box. In my opinion, these articles provide further support for the notion that the skilled artisan would be able to readily identify proteins that interact with a SOCS-box-containing protein for targeted intracellular degradation; and that the skilled artisan would be able to identify agents that inhibit or enhance the interaction between SOCS-box-containing proteins and elongin C, and further determine whether such agents inhibit or promote degradation of proteins that bind to a SOCS-box-containing protein.

14. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Nicos A Nicola

Dated: 28/11/05

Nicos A Nicola AO FAA PhD

Current Positions:	Assistant Director, The Walter and Eliza Hall Institute of Medical Research NHMRC Senior Principal Research Fellow Head, Division of Cancer and Haematology Research Professor of Molecular Haematology, Melbourne University Adjunct Professor, LaTrobe University Honorary Professor, Queensland University
Joined Institute:	1977
Date of Birth:	1 June 1950
Major Research Interests	Haemopoietic Growth Factors/Cytokines Cytokine Receptors and Signalling Leukaemia Development and Treatment

TERTIARY QUALIFICATIONS

1972
BSc (Hons), First Place and First Class Honours, Melbourne University, Biochemistry, supervised by Professor SJ Leach
1976
PhD, Melbourne University, Biochemistry, supervised by Professor SJ Leach

POSITIONS HELD

1971-75
Demonstrator of Biochemistry, University of Melbourne
1975–76
CSIRO Postdoctoral Fellow, Brandeis University, Waltham, Massachusetts
1977–79
Postdoctoral Fellow, The Walter and Eliza Hall Institute of Medical Research, Melbourne.
1979–82
Senior Research Officer, WEHI
1982–86
Research Fellow, Head – JD & L Harris Laboratory for Molecular Regulators, WEHI
1986–91
Senior Research Fellow, WEHI
1991–93
Principal Research Fellow, WEHI
1991–96
Director, Cooperative Research Centre for Cellular Growth Factors
1993–
Senior Principal Research Fellow, WEHI
1996–
Head, Division of Cancer and Haematology, WEHI
Assistant Director, WEHI
1997-
Research Professor of Molecular Haematology, Melbourne University

2002-
Adjunct Professor, Faculty of Science & Technology, LaTrobe University
2004-
Honorary Professor, Queensland University

HONOURS AND AWARDS

1971–75
CSIRO Postgraduate Award, University of Melbourne
1975
CSIRO Postdoctoral Fellowship
1977
Queen Elizabeth II Postdoctoral Fellowship
1986
Gottschalk Medal of the Australian Academy of Science
1989–90
Volunteer Units Research Award of the Anti-Cancer Council of Victoria
1991
Pharmacia-LKB Biotechnology Medal of the Australian Society for Biochemistry and Molecular Biology
1993
Wellcome Australia Medal
1996
Fellow of the Australian Academy of Science
Amgen Australia Prize
1998
Governing Council Member, International Molecular Biology Network for Asia and the Pacific Rim
1999
Austin Doyle Lecturer, High Blood Pressure Research Council of Australia, AGM, Melbourne
Barbara Ells Lecturer, Victor Chang Cardiac Research Institute, Sydney
2001
ISI Australian Citation Laureate Award (11 papers published between 1981-98 which were in the 200 most cited papers in their field internationally).
2003
Prime Minister's Centenary Medal
Honorary Professorship, University of Queensland
2005
Officer in the General Division of the Order of Australia

PROFESSIONAL ACTIVITIES

Scholarly

Editorial Boards

1990–1992
Experimental Hematology
1990-
Stem Cells
Growth Factors
1991
Current Opinion in Hematology
1994–
International Journal of Hematology
1994–99
Journal of Cell Science

1994–95
Trends in Biochemical Sciences
1995–99
Cytokine and Growth Factor Reviews
1997-
Cytokines On Line
1998-
Molecular Cell Biology Research Communications
1999-
Experimental Hematology

Conference Presentations

1989
Invited Speaker, Bone Biology Workshop, New Jersey
Invited Speaker, Sapporo Cancer Seminar, Sapporo
Invited Speaker, Growth Factors International Symposium IP, Kobe
Lecture, Kyoto Prefectural University of Medicine, Kyoto
Invited Speaker, ASI/ASMR National Scientific Conference, Adelaide

1990
Invited Speaker, Lorne Protein Conference, Lorne
Invited Speaker, Third International Workshop on Cells and Cytokines in Bone and Cartilage, Davos
Invited Speaker, Hemopoietic Growth Factors Conference, Tokyo
Invited Speaker, AACR Special Conference on Chromosomal and Growth Factor Abnormalities in Leukemia, Cape Cod
Invited Speaker, Hanson Symposium, Adelaide
Invited Speaker, Combined International Society of Hematology, Boston
American Society of Hematology Annual Meeting, Boston
Lecture, Dana Farber Cancer Institute, Boston

1991
Invited Speaker, Lorne Protein Conference, Lorne
Invited Speaker, Australian Endocrine Society Annual Meeting, Lake Hume
Invited Speaker, UCLA Conference Cytokines and their Receptors, Keystone
Award Recipient, Australian Society for Biochemistry and Molecular Biology, Annual Meeting, Canberra
Invited Speaker, Blood Cell Growth Factors: Their Present and Future use in Hematology and Oncology, Beijing
Invited Speaker, Arolla Workshop: From Receptor to Gene, Arolla
Invited Speaker, CIBA Symposium 167, Polyfunctional Cytokines – IL-6 and LIF, London
Invited Speaker, 15th Bristol-Myer Squibb Symposium on Cancer Research, Seattle

1992
CRC Directors' Meeting, Sydney
Invited Speaker, International Society of Experimental Hematology, Annual Meeting, Rhode Island
Lecture, Pharmacia-LKB Biotechnology Medal Lecture, University of Sydney, Sydney
Lecture, Pharmacia-LKB Biotechnology Medal Lecture, Queensland University, Queensland
Invited Speaker, Hanson Symposium, Adelaide
Invited Speaker, Australian & New Zealand Society of Immunology, Annual Meeting, Auckland

1993
Invited Speaker, Bioscience Forum 93, Osaka

Assigner, NH&MRC Assigners' Panel, Canberra
CRC Director – CRC Directors' Meeting, Brisbane
Invited Speaker, March Foundation Symposium, Madrid
Discussant, Sandoz Clinical Trial Meeting, Basel
Invited Speaker, Australian Society for Medical Research Annual Meeting, Adelaide
Session Chairman and Invited Speaker, Joint Meeting of the Australasian Society for Immunology and International Congress of the Society for Leukocyte Biology, Sydney
Invited Speaker, Association of Regulatory and Clinical Scientists Conference, Brisbane

1994

Invited Speaker, Training Course, UICC (International Union Against Cancer), Anti-Cancer Council of Victoria, Ludwig Institute for Cancer Research, Melbourne
Invited Speaker, Growth Factor Session of Neurosciences Towards 2000 Conference, Melbourne
Invited Speaker, Transfusion Medicine in Obstetrics and Neonatology Conference, Melbourne
Invited Speaker, Growth Factors and Genes in Myogenesis, St Vincent's Medical Research Institute, Melbourne
Invited Speaker, Seminar, Cooperative Research Centre for Biopharmaceuticals, The Garvan Institute of Medical Research, Sydney
Invited Speaker, New York Academy of Sciences Conference on Receptor Activation, Orlando
Invited Speaker, Hanson Symposium, Hanson Centre for Cancer Research, Adelaide
Invited Speaker, Australian Society for Medical Research Annual Conference, World Trade Centre, Melbourne

1995

Invited Speaker, 1st Annual Curtin Conference, Cell Signalling: From Membrane to Nucleus, Canberra
Invited Speaker, Volunteer Unit's Annual Meeting, Anti-Cancer Council of Victoria, Melbourne
Assigner, National Health and Medical Research Council Assigners' Panel, Canberra
CRC Director, Cooperative Research Centre Directors' Meeting, Melbourne
Invited Speaker, Seminar, Monash Medical Centre, Melbourne
Invited Speaker, Horizons of Science Forum Conference, University of Technology, Sydney
Speaker, International Society of Experimental Hematology – Annual Meeting, Düsseldorf
Invited Speaker, 7th FAOB Conference, Sydney

1996

Invited Speaker, Lorne Cancer Conference, Lorne
Invited Speaker, CIBA Conference, Molecular Basis of Cellular Defence Mechanisms, Melbourne
Invited Speaker, Cytokines in Bone Marrow Transplantation, Sydney
NH&MRC Assigners' Panel, Canberra
CRC Directors' Meeting, Sydney
Invited Speaker, Cytokine Receptors and Signal Transduction, Annual Inflammation Symposium, Sydney
Invited Speaker, Interferons and Cytokines, Saudi Arabia
Invited Speaker, 3rd Symposium on Haemopoietic Growth Factors, Tokyo
Invited participant, AMRAD-CHUGAI Scientific Meeting, Tsukuba and Gotemba
Invited Speaker, International Symposium for Stem Cell Regulation, Tokyo
Invited Speaker, Combined ASBMB/ASPP. Annual Scientific Meeting, Canberra
Invited Speaker, Australian Vascular Biology Society, 4th Annual Scientific Meeting, Marysville
Scientific Committee Meeting, 1st Australian Health Industry Expo, Sydney
Invited Seminar, Institute of Reproduction and Development, Monash Medical Centre
Invited Speaker, CRC-CGF Workshop on Cytosensor Technology
Invited Speaker, Hanson Symposium "Molecular Mechanisms of Oncogenesis," Adelaide
Invited Speaker, Satellite Symposium on Cell Signalling, Adelaide

1997

Invited Seminar, Microbiology Dept, Melbourne University
Assigners' Panel, NHMRC, Canberra
Induction into Australian Academy of Science, Canberra
Invited Seminar, QIMR, Brisbane
Invited participant, 1st Meeting of International Molecular Biology Network and 4th IMSUT-IMBG Symposium, Tokyo
Invited Speaker, IMSUT, Tokyo
Invited Speaker, Chugai Institute of Molecular Medicine, Tsukuba
Invited Speaker, 'From the Laboratory to the Clinic,' Trinity College, Oxford
Invited Speaker, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford
Invited Participant, 'Meeting to discuss development of a not-for-profit company to develop tropical disease products,' The Wellcome Trust, London
Plenary Speaker, 'The Myelodysplastic Syndromes,' The McCarthy Foundation, Detroit
Regional Grants Interview Committee, NHMRC, Perth
Invited Chairman, Chugai Scientific Symposium, Gotemba, Japan

1998

Invited Speaker, DNA Science Workshop for Secondary School Teachers, Melbourne
Invited Speaker, 'Reversible Associations in Structural and Molecular Biology,' RASMB, Melbourne
Plenary Speaker, '11th Symposium of Molecular Biology of Hematopoiesis,' Bormio, Italy
Plenary Speaker, '10th Anniversary Meeting of the British Cytokine group,' London
Invited Speaker, 'Current Issues in Hemopoiesis,' ASH/ASBT Annual Meeting, Sydney
Invited Speaker, 'Signal Transduction and Subcellular Compartmentalization,' Victor Harbour, South Australia
Invited Speaker, 3rd Australian Peptide Conference, Laguna Quays, Queensland
Invited Lecture, Centre for Immunology, University NSW and St Vincent's Hospital, Sydney

1999

Plenary Speaker, International Society for Experimental Hematology Annual Meeting, Monte Carlo
Plenary Speaker, 64th Cold Spring Harbor Symposium 'Signaling and Gene Expression in the Immune System', Cold Spring Harbor, New York
Invited Speaker, 1st Meeting of the Governing Council of A-IMBN, Tsukuba, Japan
Invited Speaker, 1st A-IMBN Conference 'Frontiers in Molecular Biology', Singapore
Plenary Speaker, French Society of Immunology Annual Meeting, Lille, France
Invited Lecture, St Vincent's Institute of Medical Research, Melbourne
Invited Participant, Baker Institute Retreat, Daylesford
Invited Lecture, Ivanhoe Grammar School
Austin Doyle Lecturer, High Blood Pressure Research Council of Australia, AGM, Melbourne

2000

Invited Speaker, Brisbane BioIndustry Breakfast, Brisbane.
Invited Discussant, National Innovation Summit, Melbourne.
Invited Speaker, 2nd International Workshop on Cytokine Signaling ,Aachen, Germany.
Invited Speaker, 10th Queenstown Molecular Biology Meeting, Queenstown, NZ.
Invited Speaker and Chairperson, International Congress on Differentiation and Cell Biology, Gold Coast
Invited Speaker, Kobe Symposium:GP130 Cytokines in Health and Disease, Kobe.
Invited Speaker, 'Molecular Biology and Medicine on Infectious and Immunological Diseases', Osaka
Invited Speaker, Monash Institute for Reproduction and Development, Melbourne
Barbara Ells Lecturer, Victor Chang Cardiac Research Institute, Sydney
Invited Speaker, 3rd Joint Meeting ICS/ISICR, Amsterdam
Invited Speaker, Biotech 2000 Conference, Victor Chang Cardiac Research Institute, Sydney

2001

Invited participant, A-IMBN symposium and Governing Council Meeting, Tokyo

Awardee, ISI Symposium 'Honouring Excellence in Australian Research' AAS Dome, Canberra
Invited Plenary speaker, ISICR Meeting, Cleveland,Ohio
Invited Speaker, 'Comprehensive Cancer Research Centre: Biology to Bedside' Brisbane Aug 29-30
Invited Speaker, South East Asian Biomolecular Research Training Programme Workshop, Couran Cove, Qld
Invited Speaker, Monash University Biochemistry Department Seminar Series
Invited Speaker, Opening Conference Rommelare Institute, Ghent Sept 13-14
Invited Speaker, A-IMBN symposium, Taiwan
Invited Speaker, RMIT Health Sciences, Bundoora

2002
Invited Plenary Speaker, Gordon Conference on Neurobiology, Hong Kong (Jun)
Invited Speaker, Keystone Meeting, Keystone, Colorado, Jan 2002
Invited Plenary Speaker, COMBIO 2002, Sydney (Sept)
Keynote Speaker, Licensing Executives Soc. Of Aust and NZ annual meeting, Werribee (Apr)
Invited Speaker, Institute of Knowledge Development, Managing Business Issues Series, Melb (Apr)
Invited Speaker, 2nd Awaji Intnl Forum on Infection and Immunity, Awaji (Aug)
Invited Speaker, Health and Medical Research Conference, Melbourne (Nov)
Invited Speaker, Prince Henry's Institute, Melbourne (Jul)
Invited Speaker, QIMR Annual Conference, Gold Coast (Sept)
Invited Speaker, Combined ASI/SCIL Conference, Brisbane (Dec)

2003
Invited Speaker and Chairman, Joint Session Lorne Protein and Cancer Conferences, Lorne (Feb)
Invited Speaker and Chairman, Japan/Australia Cancer Meeting, Healesville (Feb)
Invited Speaker, Hunter Valley Cell Biology Meeting (Apr)
Invited Speaker, Opening conference, IMCB, Brisbane (May)
Invited participant, National Centre in HIV Virology Research, Strategic Planning Day (Feb)
Invited Speaker and Chairman, International Conference on Cellular Engineering, Bondi (Aug)
Plenary Speaker, NSW ASMR conference, Sydney (June)
Invited Plenary Speaker, International Society for Interferon and Cytokine Research Annual Meeting, Cairns (Oct)

2004
Invited Plenary Speaker, New Era for Gene Medicine, Tokyo (Mar)
Speaker, NHMRC Program Grant Consultations, Sydney, Brisbane, Adelaide, Canberra, Melbourne (Jul)
Invited Plenary Speaker and Chair, Haematology Society of A&NZ, Melbourne (Oct)
Invited Speaker, Symposium of Australian Academy of Technological Sciences and Engineering, Adelaide (Nov)
Invited Speaker, ASMR annual conference, Sydney (Nov)

2005
Invited Speaker, IMB conference (April)
Invited Speaker, Medical Research Week, Melbourne (Jul)
Invited Speaker, 7th World Congress on Inflammation, Melbourne (Aug).
Invited Speaker, Japan Australia Collaboration in Biomedicine, Aichi, Japan (Sept)
Invited Speaker, 'Signalling Networks', Barossa Valley (Nov).

Invited Seminar, Baker Institute (Sept)

Administrative

External Committees

1991–96
Management and Scientific Committees for CRC-Cellular Growth Factors
1992–96
NH&MRC Assigners' Panel
CRC-Medical Science and Technology Section Committee
1994–95
Appointments and Promotion Committee, Ludwig Institute, Melbourne
1994–2001
Appointments and Promotions Committee, Baker Institute, Melbourne
1995
Australia Prize Selection Committee
1995–
Scientific Advisory Board, Hipple Cancer Center, Ohio
1997
Chairman, Scientific Committee, CRC-Cellular Growth Factors
Research Committee, NH&MRC
RGIC, NH&MRC
Royal Adelaide Hospital Campus Review Committee
Task Force Committee, International Molecular Biology Network
IMBN Expert Commission for Molecular Biology Needs for Asia and the Pacific Rim
1998-
Medical Review Committee – J.P. McArthy Foundation, Detroit, Michigan
Board, Prince Henry's Medical Research Institute
Research Committee, NH&MRC
Governing Council of A-IMBN
Scientific Committee, International Society for Experimental Hematology Annual Meeting 1999
1999-2002
Australian Academy of Science, Sectional Committee 8, Biochemistry, Molecular Biology and Immunology
Scientific Committee, International Society for Experimental Hematology Annual Meeting 2000
Biotechnology Consultative Group (BIOCOG) reporting to the five federal ministers.
Biotechnology Strategic Plan Steering Committee, advising Department of State and Regional Development, Victorian Government.
2000-2003
Research Committee (RC) of NHMRC
RC executive
Chair, Industry Committee of RC
2001-
Scientific Advisory Board, Institute of Molecular Bioscience, Qld.
Working Group 3, Pharmaceutical Manufacturers' Association Action Agenda
2002-
Scientific Advisory Board, Bio21
Scientific Advisory Board, CRC Chronic Inflammatory Diseases
Scientific Advisory Board, Queensland Institute for Medical Research
2003-
Research Committee (RC) of NHMRC
RC executive
Chair, Programs Committee of RC
2005
Australian Academy of Science, Sectional Committee 8
NHMRC RORA Working Group

WEHI Committees

1990–96
Chairman, Internal Finance and Advisory Committee
Library Committee
Faculty Committee
1992–96
Unit Heads Committee
1997-
Chairman, Technology Advisory Committee
Parkville Bioinformatics Committee
Senior Advisory Committee
Senior Administrative Committee
Senior Faculty Committee
Faculty Commercialization Committee
Executive Commercialisation Committee

Industry Consulting

1990-
AMRAD Corporation, Melbourne
1995-
Chugai Corporation, Japan
2000-
Quintessential Sciences Inc.
co-founder, Murigen Inc.
2004
Biota
2005
CSL

Public Activities

1995
Horizons of Science Forum, Sydney
1995-
Submissions to Industry Commission Reports on Research and Development, Innovation, Health andMedical Research Review and CRC programme
1999.
Lecture on Biotechnology, Ivanhoe Grammar School
Lecture on Biotechnology, Federal Dept. Health and Aged Care
2000.
Invited Speaker, Brisbane BioIndustry Breakfast, Brisbane.
Invited Discussant, National Innovation Summit, Melbourne
Chairman, Biotechnology Australia Intellectual Property Symposium, Adelaide
Invited Speaker, RICH Symposium of ASMR
Invited Speaker, BIOTECH 2000, Sydney
2002
Invited speaker, Institute of Knowledge Development, Business Issues Breakfast Series, Melbourne
Business Breakfast, Biotechnology Course Consultations, Box Hill TAFE
2003

Invited participant, NIH IP Policy Contact Group, Canberra
Invited participant, National Centre in HIV Virology Research, Strategic Planning Day (Feb)

TEACHING AND SUPERVISION 1990–1996

Tertiary

1987–91
DJ Hilton, PhD, Characterization of LIF and its Cell Surface Receptor
1992–95
WJ McKinstry, PhD, Molecular Analysis of Factors Active on Haemopoietic Stem Cells
1993–96
A Smith, PhD, Cellular Signalling by the GM-CSF Receptor β-chain
1996-98
Kelly Maxwell, PhD
1997.
Ruth Freeman, BSc (Hons)
2001-
Ruth Columbus, PhD
2002-
Seth Masters, PhD
David DeSouza (BSc Hons)
Marlyse DeBrincat, PhD

Post-doctoral

1990–
DJ Hilton
P Lock
Y Zhang
J-G Zhang
R Starr
C McFarlane
S Nicholson
M Baca
A Roberts

GRANTS AND CONTRACTS AWARDED 1990–

1990–
~$100,000 pa, NIH 2ROI-CA-22556, Differentiation of Granulocytes and Macrophages
$300,000 pa, AMRAD, Haemopoietic Growth Factors
$50,000 pa, JD & L Harris Trust, General
1991–2004
$30,000 pa, Philip Bushell Trust Equipment Grant
$2m pa, Cooperative Research Centres Grant, Growth Factors
1996-
$1.5m pa AMRAD grants (SOCS, LIF/IL-6, NR4,NR6)
2001-2005
US200,000pa NIH 2ROI-CA-22556, Differentiation of Granulocytes and Macrophages
2003-2007
$2.75m pa NHMRC New Program Grant (CIA)
2003

AMGEN research grant US$75,000

2005-2009
US250,000pa NIH 2ROI-CA-22556, Differentiation of Granulocytes and Macrophages, **Merit Award**

PUBLICATIONS

Refereed Primary Publications

1975

1. Appleby CA, NICOLA NA, Hurrel JGR, Leach SJ. Characterization and improved separation of soybean leghemoglobins. Biochem 14: 4444-4450, 1975
2. NICOLA NA, Minasian EM, Appleby CA, Leach SJ. Circular Dichroism studies of myoglobin and leghemoglobin. Biochem 14: 5141-5149, 1975

1976

3. Hurrel JGR, NICOLA NA, Broughton WJ, Dilworth MJ, Minasian EM, Leach SJ. Comparative structural and immunochemical properties of leghemoglobins. Eur J Biochem 66: 389-399, 1976
4. NICOLA NA, Leach SJ. Interpretations and applications of thermal difference spectra of proteins. Int J Prot Pept Res 8: 393-415, 1976

1977

5. NICOLA NA, Leach SJ. The structural basis of heme reactivity in myoglobin and leghemoglobin: Thermal difference spectra. Biochem 16: 50-58, 1977
6. NICOLA NA, Leach SJ. Structural rearrangements due to ligand binding and haem replacement in myoglobin and leghaemoglobins. Eur J Biochem 78: 133-140, 1977

1978

7. NICOLA NA, Fulmer AW, Schwartz AW, Fasman GD. High resolution proton magnetic resonance spectroscopy of histones and histone-histone complexes in aqueous solution. Biochem 17: 1779-1785, 1978
8. NICOLA NA, Burgess AW, Metcalf D, Battye FL. Separation of mouse bone marrow cells using wheat germ agglutinin affinity chromatography. Aust J Biol Med Sci 56: 663-679, 1978
9. NICOLA NA, Metcalf D, Johnson GR, Burgess AW. Preparation of colony stimulating factors from human placental conditioned medium. Leukemia Res 2: 313-322, 1978

1979

10. NICOLA NA, Kristjansson J Jr, Fasman GD. Interaction of poly (L-lysine) and copolymers of lysine with immobilized DNA. Arch Biochem Biophys 193: 204-212, 1979
11. NICOLA NA, Burgess AW, Metcalf D. Similar molecular properties of granulocyte-macrophage colony-stimulating factors produced by different mouse organs in vitro and in vivo. J Biol Chem 254: 5290-5299, 1979
12. NICOLA NA, Metcalf D, Johnson GR, Burgess AW. Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54: 614-627, 1979

1980

13. NICOLA NA, Burgess AW, Staber FG, Johnson GR, Metcalf D, Battye FL. Differential expression of lectin receptors during hemopoietic differentiation: Enrichment for granulocyte-macrophage progenitor cells. J Cell Physiol 103: 217-237, 1980
14. McCarthy JH, NICOLA NA, Szelag G, Garson OM. Studies on eosinophil colonies grown from leukemic and non-leukemic patients. Leukemia Res 4: 415-426, 1980

15. Burgess AW, Metcalf D, Russell SHM, NICOLA NA. Granulocyte/macrophage-, megakaryocyte-, eosinophil- and erythroid-colony-stimulating factors produced by mouse spleen cells. Biochem J 185: 301-314, 1980

16. Morstyn G, NICOLA NA, Metcalf D. Purification of hemopoietic progenitor cells from human marrow using a fucose-binding lectin and cell sorting. Blood 56: 798-805, 1980

1981

17. Morstyn G, NICOLA NA, Metcalf, D. Separate actions of different colony stimulating factors from human placental conditioned medium on human hemopoietic progenitor cell survival and proliferation. J Cell Physiol 109: 133-142, 1981

18. von Melchner H, NICOLA NA. Modulation by serum from irradiated and marrow transplanted mice of hemopoietic regeneration in mouse spleen organ cultures. Exp Hematol 9: 674-683, 1981

19. NICOLA NA, Metcalf D. Biochemical properties of differentiation factors for murine myelomonocytic leukemic cells in organ conditioned media - separation from colony-stimulating factors. J Cell Physiol 109: 253-264, 1981

20. NICOLA NA, Metcalf D, von Melchner H, Burgess AW. Isolation of murine fetal hemopoietic progenitor cells and selective fractionation of various erythroid precursors. Blood 58: 376-386, 1981

21. Burgess AW, Bartlett PF, Metcalf D, NICOLA NA, Clark-Lewis I, Schrader JW. Granulocyte-macrophage colony-stimulating factor produced by an inducible murine T-cell hybridoma: molecular properties and cellular specificity. Exp Hematol 9: 893-903, 1981

22. Vadas MA, Dessein A, NICOLA NA, David JR. In vitro enhancement of the helminthotoxic capacity of human blood eosinophil. Aust J Exp Biol Med Sci 59: 739-741, 1981

1982

23. NICOLA NA, Metcalf D. Analysis of purified fetal liver hemopoietic progenitor cells in liquid culture. J Cell Physiol 112: 257-264, 1982

24. Dessein AJ, Vadas MA, NICOLA NA, Metcalf D, David JR. Enhancement of human blood eosinophil cytotoxicity by semi-purified eosinophil colony-stimulating factor(s). J Exp Med 156: 90-103, 1982

25. NICOLA NA, Johnson GR. The production of committed hemopoietic colony-forming cells from multipotential precursor cells in vitro. Blood 60: 1019-1029, 1982

26. Burgess AW, Knesel J, Sparrow LG, NICOLA NA, Nice EC. Two forms of murine epidermal growth factor: Rapid separation by using reverse-phase HPLC. Proc Natl Acad Sci (USA) 79: 5753-5757, 1982

27. Metcalf D, NICOLA NA. Autoinduction of differentiation in WEHI-3B leukemia cells. Int J Cancer 30: 773-780, 1982

28. Burgess AW, NICOLA NA, Johnson GR, Nice EC. Colony-forming cell proliferation: A rapid and sensitive assay system for murine granulocyte and macrophage colony stimulating factors. Blood 60: 1219-1223, 1982

1983

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Regulation of Jak2 through the Ubiquitin-Proteasome Pathway Involves Phosphorylation of Jak2 on Y1007 and Interaction with SOCS-1

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The family of cytoplasmic Janus (Jak) tyrosine kinases plays an essential role in cytokine signal transduction, regulating cell survival and gene expression. Ligand-induced receptor dimerization results in phosphorylation of Jak2 on activation loop tyrosine Y1007 and stimulation of its catalytic activity, which, in turn, results in activation of several downstream signaling cascades. Recently, the catalytic activity of Jak2 has been found to be subject to negative regulation through various mechanisms including association with SOCS proteins. Here we show that the ubiquitin-dependent proteolysis pathway is involved in the regulation of the turnover of activated Jak2. In unstimulated cells Jak2 was monoubiquitinated, and interleukin-3 or gamma interferon stimulation induced polyubiquitination of Jak2. The polyubiquitinated Jak2 was rapidly degraded through proteasomes. By using different Jak2 mutants we show that tyrosine-phosphorylated Jak2 is preferentially polyubiquitinated and degraded. Furthermore, phosphorylation of Y1007 on Jak2 was required for proteasomal degradation and for SOCS-1-mediated downregulation of Jak2. The proteasome inhibitor treatment stabilized the Jak2–SOCS-1 protein complex and inhibited the proteolysis of Jak2. In summary, these results indicate that the ubiquitin-proteasome pathway negatively regulates tyrosine-phosphorylated Jak2 in cytokine receptor signaling, which provides an additional mechanism to control activation of Jak2 and maintain cellular homeostasis.

Most cytokines that regulate the growth and differentiation of immune and hematopoietic cells function through transmembrane receptors belonging to the cytokine receptor superfamily (19, 44). The binding of cytokines to their cognate receptors leads to dimerization or oligomerization of the receptor chains and activation of the receptor-associated Janus (Jak) family of tyrosine kinases. Jak kinases mediate essential and nonredundant functions in cytokine signaling, and individual Jaks are selectively activated by various cytokine receptors. For example, Jak2 is required for erythropoietin, interleukin-3 (IL-3), and gamma interferon (IFN-γ) signal transduction (39, 45). Jaks associate with the membrane-proximal regions of cytokine receptors, and ligand-induced aggregation of the receptor chains allows auto- and transphosphorylation of Jaks on critical tyrosine residues within the activation loop of the kinase domain. Activation of Jaks results in phosphorylation of a number of signaling proteins, such as the signal transducers and activators of transcription (STATs), phosphatidylinositol 3-kinase, and Shc, and leads to activation of intracellular signaling pathways and expression of target genes.

Regulation of Jak activity is a critical point in the modulation of cytokine responses, and recently several mechanisms for regulating Jak activation have been described. Intramolecular interactions control the activity of the tyrosine kinase domain in Jak kinases (41, 53). The SH2 domain-containing tyrosine phosphatases SHP-1 and SHP-2 have been shown to have both stimulatory and inhibitory effects on cytokine receptor signaling (23). An important mechanism for negative regulation of cytokine signaling is mediated through members of the recently identified SOCS (suppressor of cytokine signaling) family of proteins (2, 5). The SOCS family consists of eight members that have highly specialized functions in regulation of cytokine signaling. One of the family members, SOCS-1, also termed JAB (Jak binding protein) or SSI-1 (STAT-induced STAT inhibitor 1), was identified through its ability to inhibit IL-6 signal transduction and bind to Jak2 (11, 34, 46). SOCS-1 has been shown to have a crucial function in regulation of Jak2 activation, IFN-γ responses, and T-cell differentiation (1, 3, 30).

The SOCS proteins contain a central SH2 domain, which interacts either with the autophosphorylation site tyrosines in Jaks or with the phosphorylated tyrosine residues in cytokine receptors (36, 40). The hallmark of the SOCS family is a C-terminal homology domain referred to as the SOCS box, which has been found in a large number of proteins: WD-40 repeat-containing proteins, SPRY domain-containing proteins, ankyrin repeat-containing proteins, and small GTPases (18). The SOCS boxes of SOCS-1 and SOCS-3 were found to mediate interaction with the elongin B/C complex, and the SOCS box contains a conserved elongin B/C binding motif (BC box) in the N terminus (57). The elongin B/C complex was initially

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identified as a component of the multiprotein von Hippel-Lindau tumor suppressor E3 ligase complex, which also contains RING finger protein Rbx1 as a bridging factor and Cullin-2 (10, 22). The interaction between the SOCS box and elongins B and C implicates the ubiquitin-proteasome pathway in regulation of SOCS function and protein turnover. SOCS-3 is rapidly degraded through the proteasome pathway, suggesting that the interaction with elongins B and C directs the multiprotein complex to proteasomes for degradation (57). However, for SOCS-1, the SOCS box and interaction with elongins B and C is reported to inhibit degradation of SOCS-1 (21, 35). The possible role of the SOCS-elongin B/C complex in regulation of Jak kinases in cytokine signaling is currently not known.

The ubiquitin-proteasome pathway mediates specific degradation of regulatory proteins and plays an important role in controlling a variety of cellular functions such as DNA repair, cell cycle control, antigen presentation, intracellular translocation of proteins and apoptosis (6). Conjugation of ubiquitin to the substrate proceeds in three distinctive steps: first, ubiquitin is activated by the ubiquitin-activating enzyme (E1) in an ATP-dependent reaction; second, activated ubiquitin is transferred onto a ubiquitin carrier protein (E2); third, the ubiquitin moieties are transferred onto a ubiquitin ligase (E3), which catalyzes the covalent modification of lysine residues on the substrate with the ATP-activated ubiquitin. Polyubiquitinated proteins are then recognized by the 19S complex of the 26S proteasomes and degraded into short peptides with ubiquitin recycled via the action of isopeptidases (48).

Several lines of evidence have also implicated the ubiquitin-proteasome pathway in regulation of cytokine receptor signaling. Various components of the ubiquitin pathway are regulated by cytokine-induced signals. For example, IFNs have been shown to induce the expression of the UbcH family of ubiquitin-conjugating enzymes, and different isozymes of deubiquitinating enzymes are induced by cytokines in a Jak-dependent manner (8, 37). Proteasome inhibitors have been shown to prolong activation of the Jak/STAT pathway in response to IL-2, IL-3, growth hormone (GH), ciliary neurotrophic factor, IFN, and erythropoietin stimulations (4, 14, 24, 29, 49, 50, 56). The underlying molecular mechanisms by which this occurs are still, however, poorly understood. The tyrosine-phosphorylated forms of STAT4, STAT5, and STAT6, but not other STATs, have been shown to be stabilized by proteasome inhibitors, but ubiquitination of these proteins was not detected (51). Cell type-dependent differences, for example, in expression of critical regulatory proteins, may also account for the specificity of the ubiquitin-proteasome pathway. For example, STAT1 becomes ubiquitinated after IFN-γ stimulation in HeLa cells (24), and tyrosine-phosphorylated Stat1 has been found to be stabilized by proteasome inhibitors in fibroblasts without any indication of ubiquitination (16). In addition, certain cytokine receptors such as EpoR, GHR, IL-2Rβ, and IL-9R have been shown to become ubiquitinated, and proteasomes control the turnover of these receptors (50).

Here we present evidence that the ubiquitin-proteasome pathway directly regulates the activated form of Jak2. We show that Jak2 is ubiquitinated in vivo and in vitro and that IL-3 and IFN-γ stimulation increases the accumulation of polyubiquitinated forms of Jak2 that are stabilized by proteasome inhibitor treatment. Tyrosine phosphorylation was found to be a requirement for efficient ubiquitination and proteasomal degradation of Jak2. These findings led us to investigate the role of SOCS proteins in this regulation, and proteasome inhibitors were found to stabilize the interaction between Jak2 and SOCS-1. Expression of SOCS-1 enhanced proteasome-dependent degradation of the activated form of Jak2. Importantly, ubiquitination and protein levels of Jak2YF, a Jak2 mutant with a Y1007F mutation that fails to interact with SOCS-1, were not affected by coexpression of SOCS-1. Thus, the Jak2 protein is a target for ubiquitin-proteasome-mediated regulation of cytokine signaling, in which tyrosine phosphorylation of Jak2 and the interaction with SOCS-1 function as regulatory mechanisms.

MATERIALS AND METHODS

Antibodies and reagents. The following antibodies were used: antiphosphotyrosine clone 4G10 (Upstate Biotechnology, Lake Placid, N.Y.), a polyclonal anti-Jak2 antibody, kind gift from J. N. Ihle (45), an anti-influenza virus hemagglutinin (HA) epitope antibody (clone 16B12; Berkeley Antibody, Richmond, Calif.), antiubiquitin mouse monoclonal antibody mAb-Ubi-1 (Sigma-Aldrich RBI), and anti-SOCS-1 and anti-SOCS-3 monoclonal antibodies (57). Recombinant murine IL-3 was purchased from PeproTech (London, England), and recombinant human IFN-γ was purchased from Immugenex. Proteasome inhibitors MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal; Z-LLL-CHO), *clasto*-lactacystin-β-lactone (β-lactacystin), and ubiquitin aldehyde were purchased from Calbiochem-Novabiochem (Darmstadt, Germany); MG132 and β-lactacystin were dissolved in dimethyl sulfoxide. Biotinylated antimouse and antirabbit antibodies were purchased from Dako A/S, and streptavidin-biotinylated horseradish peroxidase was purchased from Amersham Pharmacia Biotech. Rabbit reticulocyte lysates were purchased from Promega (Madison, Wis.), and ubiquitin, hexokinase, D-glucose, and ATP for the in vitro ubiquitination reaction were purchased from Sigma. Pervanadate was prepared as follows: 100 μl of 100 mM Na_3VO_4 was mixed with 88 μl of Tris-buffered saline (TBS) and 12 μl of 30% H_2O_2. The mixture was used within 5 min of preparation.

Cell culture. Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin/ml, and 100 μg of streptomycin/ml. IL-3-dependent myeloid progenitor 32D cells were maintained in RPMI medium (Gibco-BRL) supplemented with 4% WEHI supernatant, 10% FCS, and antibiotics. Starvation was performed by washing the cells with phosphate-buffered saline (PBS) prior to incubation in medium containing 0.5% FCS for 12 to 14 h.

Plasmid construction and transfections. The plasmids expressing Jak2WT-HA and Jak2KN-HA have been described previously (41). Jak2KN-HA contains a K882E substitution in the lysine involved in the phosphotransfer reaction. The Y1007F Jak2 mutant is identical with Jak2WT-HA except for the Y1007F substitution created by direct PCR mutagenesis using the following primers: 5'TGC CGC AGG ACA AAG AAT TCT ACA AAG TAA AGG AGC CA and 3'TGG CTC CTT TAC TTT GTA GAA TTC TTT GTC CTG CTG CGG CA. HA-tagged ubiquitin and His_6-ubiquitin were kind gifts from D. Bohman (EMBL Heidelberg, Germany). SOCS-1, SOCS-1ΔSB, and SOCS-3 expression plasmids have been previously described (18, 58). Cos-7 cells were transfected by electroporation using a Bio-Rad Pulse apparatus.

Pulse-chase experiments. Transfected Cos-7 cells were pretreated with proteasome inhibitor MG132 for 1 h before lysis, and the proteasome inhibitors were maintained throughout the experiments. Cells were then transferred to methionine-free and cysteine-free DMEM (Gibco-BRL) for 30 min, pulsed with 0.1 mCi of [^{35}S]methionine and [^{35}S]cysteine (ProMix; Amersham) for 15 min, and chased with DMEM followed by stimulations (see Fig. 7).

Immunoprecipitation and Western blotting. The cells were harvested in ice-cold PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 7.4], 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 20 mM NaF, 0.2 mM Na_3VO_4) supplemented with protease inhibitors. After 30 min of incubation on ice, lysates were cleared by centrifugation for 20 min at 4°C. For detection of ubiquitination in endogenous Jak2, isopeptidase activity was inhibited by addition of 20 μg of ubiquitin aldehyde/ml to the lysis buffer or, alternatively, the cells were lysed by being boiled in PBS–2% sodium dodecyl sulfate (SDS) buffer as described previously (47). The protein amount was determined by a Bio-Rad Dc protein assay kit (Bio-Rad Laboratories). Immunoprecipitations were carried

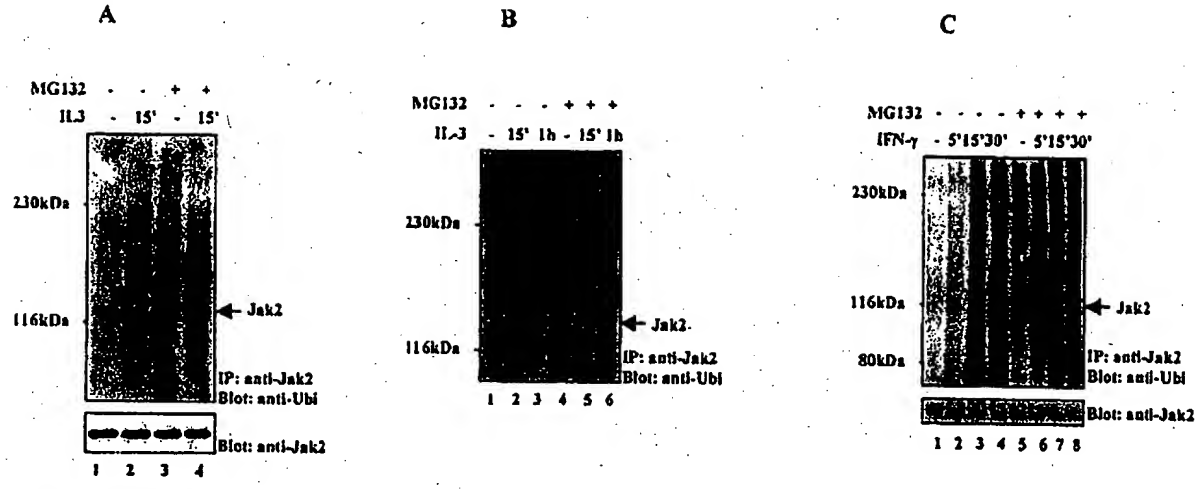

FIG. 1. Ubiquitination of Jak2 in response to cytokine stimulation. (A) 32D cells were starved overnight and pretreated with proteasome inhibitor MG132 (20 μM) or vehicle for 1 h before IL-3 (100 ng/ml) stimulation. The cells were lysed in NP-40 lysis buffer supplied with 20 μg of MG132/ml and 20 μg of ubiquitin aldehyde/ml to inhibit isopeptidase activities. Jak2 was immunoprecipitated (IP) and subjected to immunoblotting using an antiubiquitin antibody. After being stripped the filter was reblotted with an anti-Jak2 antibody. (B) 32D cells were treated as described in for panel A. Cell extracts were prepared under denaturing lysis conditions, and the immunoprecipitated Jak2 was analyzed by immunoblotting using an antiubiquitin antibody. (C) Cos-7 cells were starved overnight and pretreated with proteasome inhibitor MG132 (20 μM) or vehicle for 1 h before IFN-γ (100 ng/ml) stimulation. Cell extracts were prepared under denaturing lysis conditions, and the immunoprecipitated Jak2 was analyzed by immunoblotting using an antiubiquitin antibody. After being stripped the filter was reblotted with an anti-Jak2 antibody.

out as previously described (25). For antiubiquitin detection, immunoprecipitations were performed in the presence of 25 μM MG132 and immunoblotting was carried out as described previously (38). The stripping was performed by incubating the filters in 62.5 mM Tris-HCl–100 mM 2-mercaptoethanol–2% SDS for 45 min at 56°C followed by extensive washings and blocking with 5% nonfat dry milk in TBS.

Immunodetection. After SDS-polyacrylamide gel electrophoresis the proteins were transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell GmbH) and blocked with 5% nonfat dried milk in TBS–0.1% Tween 20. The membranes were incubated with specific antibodies diluted in TBS–0.05% Tween 20. Immunodetection was performed using the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

In vitro ubiquitination of Jak2. Jak2 was immunoprecipitated from transfected Cos-7 cells by using an anti-Jak2 antibody and purified with protein A-Sepharose beads. After extensive washes, the beads were resuspended in 30 μl of buffer containing 40 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 2 mM ATP, 5 μg of ubiquitin/ml, 12.5 μM MG132, and 20 μg of ubiquitin aldehyde/ml. To deplete endogenous ATP, hexokinase (1 mg/ml) and 2-deoxyglucose (20 mM) were also included. Five microliters of crude rabbit reticulocyte lysates was added to each reaction mixture, and the mixture was incubated for 1.5 h at 30°C. The beads were then extensively washed and resuspended in SDS loading buffer. Cell lysates from Cos-7 cells transfected with SOCS-1, SOCS-1ΔSB, or a control were prepared by washing the cells twice with ice-cold PBS and lysing them in 250 μl of lysis buffer (20 mM HEPES [pH 7.2], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 25 μM MG132, and protease and phosphatase inhibitors). Lysates were sonicated for two cycles of 30 s followed by centrifugation for 30 min. For the ubiquitination reaction, immunoprecipitated Jak2 (as described above) bound to protein A-Sepharose was resuspended in 50 μl of reaction buffer (20 mM HEPES [pH 7.2], 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mg of hexokinase/ml, 20 mM D-glucose, 25 μM MG132, 5 μg of ubiquitin/ml) containing 100 μg of cell lysates and then the suspension was incubated for 90 min at 30°C, after which the bound Jak2 was washed and resuspended in SDS loading buffer.

RESULTS

Jak2 is ubiquitinated in vivo, and its ubiquitination is regulated by cytokines. Proteasome inhibitors have been shown to prolong the Jak/STAT signaling pathway, but the underlying molecular mechanisms remain poorly understood. We wanted to investigate the role of the proteasome pathway in regulation of Jak activation and initially examined whether Jak2 is subject to ubiquitination in response to IL-3 stimulation. For these experiments, IL-3-dependent myeloid 32D cells were starved overnight and stimulated with IL-3 in the presence or absence of proteasome inhibitors. Two different proteasome inhibitors were used, MG132, which is a reversible 26S proteasome inhibitor, and β-lactacystin, which is an irreversible proteasome inhibitor that blocks the isopeptidase activities as well. 32D cells were lysed with a nondenaturing buffer containing proteasome inhibitors as well as ubiquitin aldehyde to inhibit isopeptidase activities. Jak2 was immunoprecipitated and subjected to antiubiquitin and anti-Jak2 immunoblotting. IL-3 stimulation resulted in rapid tyrosine phosphorylation of Jak2, and MG132 treatment stabilized the tyrosine phosphorylation of Jak2 as previously reported (4) (data not shown). A protein whose molecular weight corresponded to that of Jak2 was detected by the antiubiquitin antibody in both unstimulated and IL-3-stimulated 32D cells (Fig. 1A). Stripping and reblotting the filter with an anti-Jak2 antibody indicated that the protein was Jak2. Based on the molecular weight this band represented a monoubiquitinated form of Jak2. IL-3 stimulation induced a smear above the Jak2 band, which is characteristic of polyubiquitination. This smear could also be detected upon longer exposures using antiphosphotyrosine antibodies (data not shown). The presence of ubiquitin aldehyde in the lysis buffer was critical for detection of the polyubiquitinated forms of Jak2. Pretreatment of 32D cells with MG132 did not affect the level of monoubiquitinated Jak2 but increased the accumulation of polyubiquitinated Jak2. Control immunoprecipitations with antibodies against STAT1, STAT6, and PU.1

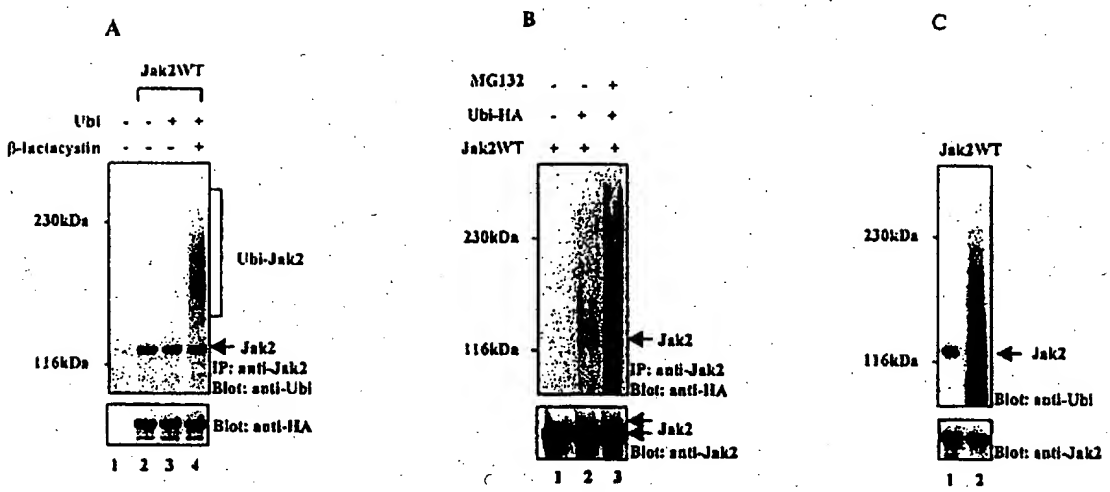

FIG. 2. In vivo and in vitro ubiquitination of Jak2. (A) Ubiquitination of Jak2 in Cos-7 cells. Cos-7 cells were transiently transfected with plasmids encoding Jak2WT-HA and ubiquitin (Ubi). The sample from lane 4 was pretreated with proteasome inhibitor β-lactacystin (10 μM) for 12 h before harvesting. Cell extracts were immunoprecipitated (IP) with an anti-Jak2 antibody and analyzed by immunoblotting with an antiubiquitin antibody. After being stripped the filter was blotted with an anti-HA antibody. (B) Detection of Jak2 ubiquitination using an anti-HA antibody. Cos-7 cells were transfected with Jak2WT (lanes 1 to 3) and Ubi-HA (lanes 2 and 3) expression plasmids, and Jak2 was immunoprecipitated with anti-Jak2 antibodies and analyzed by immunoblotting with an anti-HA antibody. Lane 3 represents the sample pretreated with MG132 (10 μM) for 3 h before harvesting. The filter was stripped and reblotted with an anti-Jak2 antibody. (C) In vitro ubiquitination of Jak2. Immunopurified Jak2WT-HA from transfected Cos-7 cells was subjected to an in vitro ubiquitination reaction. After the reaction, Western blotting was performed using an antiubiquitin antibody. Lane 1, input for the ubiquitination reaction; lane 2, immunopurified Jak2 after a 1.5-h ubiquitination reaction.

indicated that the antiubiquitin antibody detected a faint nonspecific band slightly above Jak2 (data not shown). Preclearing the lysates with irrelevant antibodies did not affect the intensity of the Jak2 band detected by antiubiquitin blotting.

Ubiquitination of Jak2 was next investigated by using a denaturing lysis protocol to inhibit the proteasome as well as the isopeptidase activities. In 32D cells IL-3 stimulation induced rapid polyubiquitination of Jak2, which decreased by 1 h (Fig. 1B). The proteasome inhibitor treatment stabilized the polyubiquitinated form of Jak2, thus suggesting that the polyubiquitinated forms of Jak2 are normally rapidly removed through the proteasome pathway. Similar results were obtained with another IL-3-dependent myeloid cell line, FDCP-1 (data not shown). We also investigated whether other cytokine signals would regulate ubiquitination of Jak2. IFN-γ stimulation induced ubiquitination of the endogenous Jak2 protein, and MG132 treatment enhanced and stabilized the polyubiquitination of Jak2 (Fig. 1C). Taken together, these data indicate that the ubiquitin-proteasome pathway is involved in regulation of Jak2 in IL-3 and IFN-γ signal transduction.

Ubiquitination of Jak2 in intact cells and in a cell-free system. Modulation of cytokine receptors by the proteasome pathway is a potential mechanism for the effects of proteasome inhibitors and for regulation of Jak2 activity. To exclude this, ubiquitination of Jak2 in Cos-7 cells, where Jaks can be activated by overexpression in a receptor-independent mechanism, was next investigated. Cos-7 cells were transiently transfected with plasmids encoding HA epitope-tagged wild-type Jak2 (Jak2WT-HA) and ubiquitin. The Jak2 protein was immunoprecipitated with an anti-Jak2 antibody and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with antiubiquitin and anti-HA antibodies. As shown in Fig. 2A, endogenous Jak2 from untransfected cells was weakly ubiquitinated (lane 1) and overexpression of Jak2WT-HA enhanced the ubiquitination of Jak2. Coexpression of the ubiquitin expression plasmid with that for Jak2WT-HA did not have any significant effect on the level of Jak2 ubiquitination, suggesting that the availability of ubiquitin is not a rate-limiting step in this reaction. After being stripped, the filter was probed with the anti-HA antibody, which indicated that the band detected by antiubiquitin was the Jak2 protein. β-Lactacystin treatment resulted in accumulation of multiubiquitinated forms of Jak2, detected as the characteristic high-molecular-weight smear. Preclearing the lysates with an irrelevant monoclonal antibody did not affect the ubiquitin signal of Jak2 (data not shown)

To confirm the ubiquitination of Jak2, Cos-7 cells were transiently transfected with Jak2WT (without the HA tag) and HA epitope-tagged ubiquitin (Ubi-HA) expression plasmids (Fig. 2B). Jak2 was immunoprecipitated with the anti-Jak2 antibody, and ubiquitination was detected by anti-HA blotting. The anti-HA antibody detected Jak2 only when Ubi-HA was cotransfected, and MG132 treatment increased the ubiquitination. The anti-HA and anti-Jak2 blots show typical laddering caused by sequentially attached ubiquitin molecules. Thus, these results confirmed that Jak2WT was monoubiquitinated in Cos-7 cells, and proteasome inhibitor treatment enhanced polyubiquitination of Jak2.

We also set up an in vitro assay to analyze if Jak2 is subject to ubiquitination in a cell-free system. Such a system was previously successfully used in demonstrating the ubiquitination of the epidermal growth factor receptor (EGFR) in vitro (28). As starting material for the reaction we used immunopurified

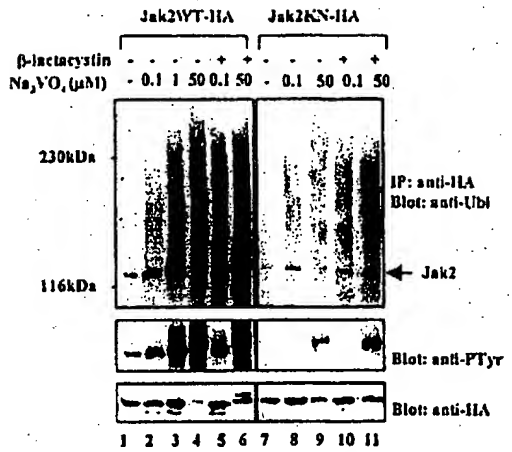

FIG. 3. Tyrosine phosphorylation of Jak2 correlates with its ubiquitination and degradation. Jak2WT-HA (left) and Jak2KN-HA (right) were transiently expressed in Cos-7 cells. After 48 h the cells were starved overnight and pretreated with proteasome inhibitor β-lactacystin (20 μM) or vehicle for 1 h before treatment with different concentrations of pervanadate for 1 h. Total-cell lysates were immunoprecipitated (IP) with an anti-HA antibody and analyzed by immunoblotting using antiubiquitin or antiphosphotyrosine (anti-PTyr) antibodies. The antiubiquitin and antiphosphotyrosine blots from Jak2WT-HA and Jak2KN-HA panels were exposed for the same time. The filters were then stripped and blotted with an anti-HA antibody (bottom).

Jak2WT from transfected Cos-7 cells, crude rabbit reticulocyte lysate, ATP, and ubiquitin. The reaction was allowed to proceed for 1.5 h. Immunoblotting with the antiubiquitin antibody demonstrated that the input Jak2 protein was already monoubiquitinated and that the in vitro ubiquitination reaction enhanced the monoubiquitinated and polyubiquitinated forms of Jak2 (Fig. 2C).

Polyubiquitination and degradation of Jak2 are regulated through tyrosine phosphorylation. Our results indicated that cytokine stimulation induced polyubiquitination of Jak2. Next, we wanted to examine directly the role of tyrosine phosphorylation in ubiquitination and degradation of Jak2. For this purpose Cos-7 cells were transiently transfected with Jak2WT-HA or kinase-negative Jak2 (Jak2KN-HA) expression plasmids (see Materials and Methods). Overexpression results in tyrosine phosphorylation and activation of Jak2WT. For these experiments the expression level of Jak2 was titrated to a level which allowed the regulation of Jak2 tyrosine phosphorylation by tyrosine phosphatase inhibitor pervanadate. The cells were treated with different concentrations of pervanadate for 1 h to induce tyrosine phosphorylation of Jak2. Jak2 was immunoprecipitated with the anti-HA antibody and analyzed by Western blotting. As illustrated in Fig. 3, pervanadate treatment induced, in a dose-dependent manner, ubiquitination of Jak2, which correlated with enhanced tyrosine phosphorylation of Jak2. Treatment of the cells with 50 μM pervanadate caused phosphorylation and polyubiquitination of Jak2 and disappearance of the monoubiquitinated form of Jak2. Interestingly, anti-HA blotting showed that 50 μM pervanadate treatment resulted in a dramatic decrease in Jak2 protein levels. Inhibition of proteasomes by β-lactacystin enhanced both polyubiquitination and tyrosine phosphorylation of Jak2 and stabilized the phosphorylated Jak2 protein (Fig. 3). Thus, these results suggest that phosphorylation of Jak2 serves as a signal for the ubiquitination reaction and for the degradation of Jak2 through the proteasome pathway.

Jak2KN was found to be monoubiquitinated at low levels in Cos-7 cells (Fig. 3). Jak2KN is not significantly tyrosine phosphorylated when overexpressed in Cos-7 cells, but a high concentration of pervanadate resulted in low levels of tyrosine phosphorylation of Jak2KN, probably due to transphosphorylation. A modest increase in mono- and polyubiquitination of Jak2KN was detected after pervanadate treatment, and β-lactacystin pretreatment enhanced ubiquitination as well as tyrosine phosphorylation of Jak2KN. Pervanadate treatment (50 μM) decreased Jak2KN protein levels slightly. These results further substantiate the correlation between tyrosine phosphorylation and ubiquitination of Jak2 and the involvement of proteasomes in regulation of the tyrosine-phosphorylated form of Jak2.

To analyze in more detail the role of tyrosine phosphorylation in regulation of Jak2 via the ubiquitin-proteasome pathway, we performed kinetic studies using pervanadate (50 μM) and proteasome inhibitor MG132 (25 μM). Cos-7 cells were transiently transfected with Jak2WT-HA (Fig. 4A) or Jak2KN-HA (Fig. 4B). Jak2 was immunoprecipitated using the anti-HA antibody and analyzed by Western blotting. As shown in Fig. 4A, pervanadate treatment rapidly increased the polyubiquitination of Jak2, with a peak at 15 min, after which the level of ubiquitination decreased and became undetectable after 2 h. Tyrosine phosphorylation of Jak2 also showed a time dependence, with pervanadate treatment stimulating the phosphorylation of Jak2, followed by a decline in the level of phosphorylation. Anti-HA blotting indicated that the decreased levels of phosphorylation and ubiquitination correlated with degradation of Jak2. Preincubation with MG132 enhanced ubiquitination and phosphorylation of Jak2. Furthermore, MG132 treatment stabilized the Jak2 protein levels, particularly the lower-mobility forms of Jak2, which are likely to represent phosphorylated forms of Jak2 proteins.

The effects of pervanadate treatment on ubiquitination, tyrosine phosphorylation, and degradation of Jak2KN and of Jak2WT showed clear differences in magnitude and slight differences in time course (Fig. 4B). Tyrosine phosphorylation of Jak2KN occurred at low levels, and polyubiquitination appeared slightly later, showing a peak at 30 min. The anti-HA Western blot indicated that, compared to those of Jak2WT, the Jak2KN protein levels were less affected by pervanadate treatment, a finding which correlates with the lower level of tyrosine phosphorylation and ubiquitination. MG132 stabilized the protein levels and enhanced both tyrosine phosphorylation and ubiquitination of Jak2KN.

SOCS-1 regulates the degradation of Jak2 through the proteasome pathway. SOCS-1 is reported to bind to the phosphorylated activation loop tyrosine in Jak2 and to inhibit the catalytic activity of Jak2 (52). The association of SOCS proteins with elongins B and C has, however, raised the possibility that the SOCS-mediated regulation of cytokine signaling may also involve the proteasome pathway (21). Our results indicated that tyrosine phosphorylation regulates the degradation of Jak2 via the ubiquitin-proteasome pathway, and therefore we

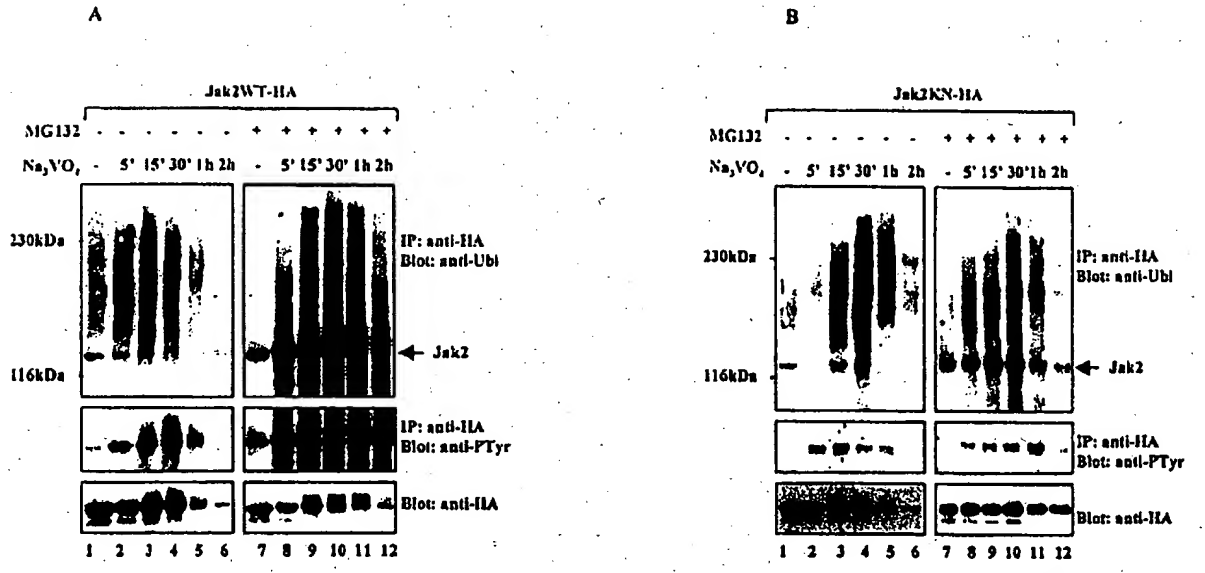

FIG. 4. Kinetics of Jak2 ubiquitination and degradation in vivo. Jak2WT-HA (A) and Jak2KN-HA (B) were transiently expressed in Cos-7 cells. After 48 h the cells were starved overnight and stimulated with pervanadate (50 μM) as indicated. Right blots (A and B), lysates from cells pretreated with proteasome inhibitor MG132 (25 μM) for 1 h before pervanadate treatment. Jak2 was assessed by immunoblotting using an antiubiquitin or antiphosphotyrosine (anti-PTyr) antibody followed by stripping and anti-HA blotting. The antiubiquitin and antiphosphotyrosine blots for Jak2WT-HA and Jak2KN-HA were exposed for the same time. IP, immunoprecipitation.

wanted to investigate whether SOCS proteins could be involved in ubiquitin-mediated degradation of Jak2. To test this hypothesis, Cos-7 cells were transiently transfected with plasmids expressing Jak2WT-HA alone or together with SOCS-1, and the cells were treated with pervanadate (25 μM) in the presence or absence of proteasome inhibitor MG132 (20 μM). Coexpression of SOCS-1 resulted in reduced levels of ubiquitinated Jak2 compared to expression of Jak2 alone (Fig. 5A).

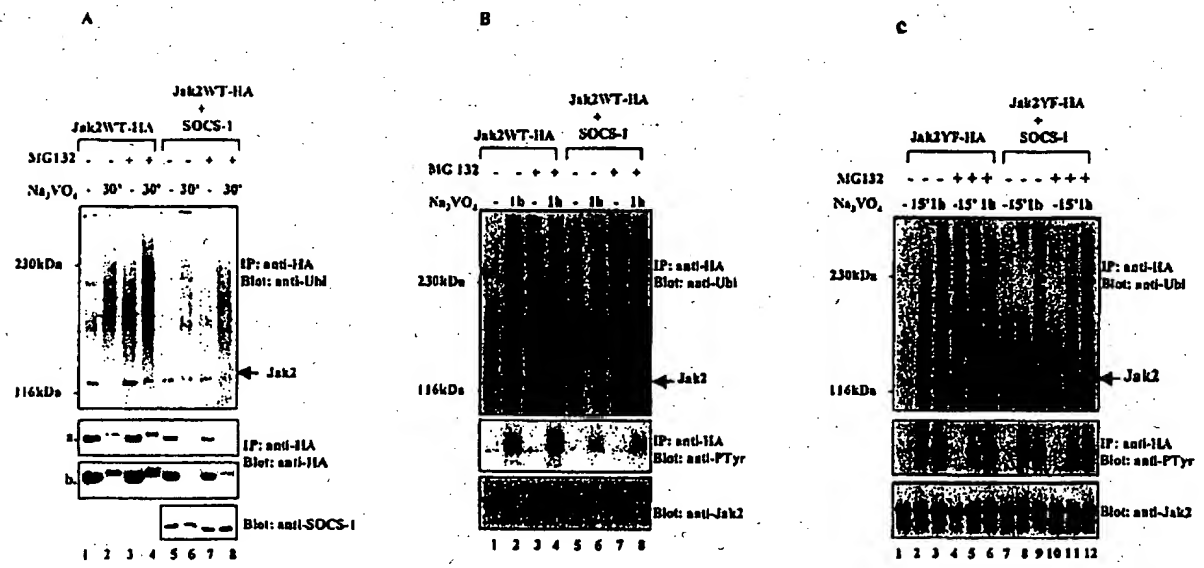

FIG. 5. SOCS-1 coexpression enhances proteasomal degradation of Jak2WT but not Jak2YF. Cos-7 cells were transiently transfected with plasmids encoding Jak2WT-HA (A and B) or Jak2YF-HA (C) either alone or together with plasmids encoding SOCS-1. After 48 h the cells were starved overnight and pretreated with proteasome inhibitor MG132 (20 μM) for 1 h before pervanadate treatment (25 μM). Jak2 was analyzed by immunoprecipitation (IP) with an anti-HA antibody followed by immunoblotting with the indicated antibodies. (A, a and b) Different exposures of the same blot. SOCS-1 protein levels were determined by Western blotting 20 μg of total-cell lysates using an anti-SOCS-1 antibody.

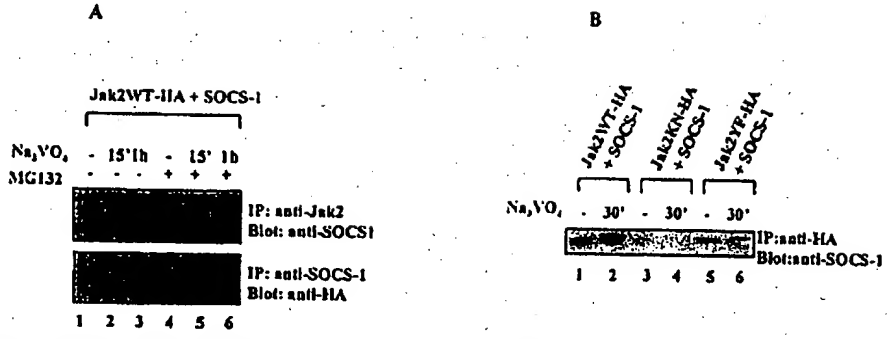

FIG. 6. Proteasome inhibitor MG132 stabilizes the interaction between Jak2WT and SOCS-1. (A) Coimmunoprecipitation of Jak2 and SOCS-1. Cos-7 cells were transfected with Jak2WT-HA and SOCS-1 as indicated and treated with MG132 and pervanadate as described for Fig. 5. Total-cell lysates were immunoprecipitated (IP) with anti-HA or anti-SOCS-1 antibodies and analyzed by immunoblotting using anti-SOCS-1 or anti-HA antibodies. (B) Coimmunoprecipitation of SOCS-1 with different Jak2 mutants. Cos-7 cells were transfected with Jak2WT-HA (lanes 1 and 2), Jak2KN-HA (lanes 3 and 4), and Jak2YF-HA (lanes 5 and 6) together with SOCS-1. Cells were pretreated with MG132 (25 μM) for 1 h before the pervanadate treatment (25 μM). After anti-HA immunoprecipitation, the filter was immunoblotted with an anti-SOCS-1 antibody.

Anti-HA immunoblotting indicated that coexpression of Jak2 with SOCS-1 caused a reduction in Jak2 protein levels (Fig. a, lanes 1 and 5), which were further decreased by pervanadate treatment. Pretreatment of the cells with MG132 partially stabilized Jak2 protein levels in the presence of SOCS-1, as shown in longer exposures of the anti-HA blot (Fig. b). These results suggest that SOCS-1 enhanced proteasomal degradation of Jak2. An anti-SOCS-1 Western blot showed that pervanadate had only a marginal effect on SOCS-1 protein levels.

To analyze the Jak2–SOCS-1 interaction in more detail, similar experiments were performed with autophosphorylation site mutant Jak2YF mutant, which has been shown to fail to interact or to interact only weakly with SOCS-1 (52). Interestingly, pervanadate treatment induced monoubiquitination and tyrosine phosphorylation of Jak2YF but only very low levels of polyubiquitnation (Fig. 5C). In striking contrast to the results obtained with Jak2WT (Fig. 5A and B), the protein levels of Jak2YF were not affected by pervanadate treatment. Furthermore, SOCS-1 expression did not have any effect on ubiquitination, tyrosine phosphorylation, or protein levels of Jak2YF (Fig. 5C).

The results in Fig. 5 suggest that autophosphorylation site Y1007 and the interaction with SOCS-1 regulate the ubiquitin-mediated degradation of Jak2. To validate this hypothesis, we analyzed the interaction between Jak2 and SOCS-1 directly. Cos-7 cells were cotransfected with Jak2WT-HA and SOCS-1, and the association between Jak2 and SOCS-1 was analyzed. As shown in Fig. 6A, pervanadate enhanced the association between SOCS-1 and Jak2 in anti-Jak2 immunoprecipitations but the amount of coimmunoprecipitated SOCS-1 decreased after 1 h of treatment. MG132 pretreatment stabilized the Jak2–SOCS-1 complex. Similar results were obtained when the experiments were performed in the reverse direction by immunoblotting SOCS-1 immunoprecipitates with anti-Jak2 for Jak2. These results, together with the finding that SOCS-1 protein levels were not affected by pervanadate treatment (Fig. 5A), suggest that MG132 inhibited the degradation of the ubiquitinated Jak2 in the Jak2–SOCS-1 complex. The interaction between SOCS-1 and Jak2 mutants was also analyzed, and, as shown in Fig. 6B, SOCS-1 showed only very weak association with Jak2KN-HA and Jak2YF-HA, suggesting that the association of SOCS-1 with Jak2 requires phosphorylation of Jak2 on tyrosine 1007.

The results obtained from Western blotting were confirmed by metabolic-labeling experiments. Cos-7 cells were transfected with Jak2WT-HA or Jak2KN-HA and Jak2YF-HA alone or together with SOCS-1 and then pulse labeled with ^{35}S in the presence of MG132. Pervanadate treatment caused a mobility shift in Jak2WT but not in Jak2 mutants (Fig. 7A). SOCS-1 coexpression decreased the tyrosine-phosphorylated slower-migrating forms of Jak2WT, while the protein levels of Jak2KN-HA and Jak2YF-HA were not affected by SOCS-1 coexpression or pervanadate treatment. A slight decrease in Jak2KN protein level was noticed after 1 h of pervanadate treatment (Fig. 7A, bottom, lane 6).

Pulse-chase experiments were conducted to investigate the effect of SOCS-1 on the half-lives of different Jak2 proteins. Cos-7 cells were transfected with different Jak2 plasmids alone or together with SOCS-1 and pulse-labeled with ^{35}S and then chased for different time periods (Fig. 7B). Jak2 was immunoprecipitated with the anti-HA antibody. After autoradiography the amount of the immunoprecipitated Jak2 protein was quantified. Jak2KN was found to have a shorter half-life than Jak2WT or Jak2YF for currently unknown reasons. However, in accordance with results shown above, the half-lives of Jak2KN-HA and Jak2YF-HA mutants were not affected by SOCS-1 while the degradation of Jak2WT was accelerated in the presence of SOCS-1.

We wanted to validate the specificity of SOCS-1 in the ubiquitin-mediated degradation of Jak2 by analyzing the effect of related protein SOCS-3 on Jak2 protein turnover in Cos-7 cells. Cotransfection of SOCS-3 had no effect on Jak2 protein levels (Fig. 8). Pervanadate treatment caused tyrosine phosphorylation of SOCS-3, as shown by detection of the low-mobility forms of the protein (7), and a slight reduction of SOCS-3 protein, which was stabilized by MG132 treatment.

The SOCS box is required for ubiquitination of Jak2. The SOCS box of SOCS-1 interacts with elongins B and C and may target the bound molecules through proteasomal degradation (57). To determine the role of the SOCS box in the ubiquiti-

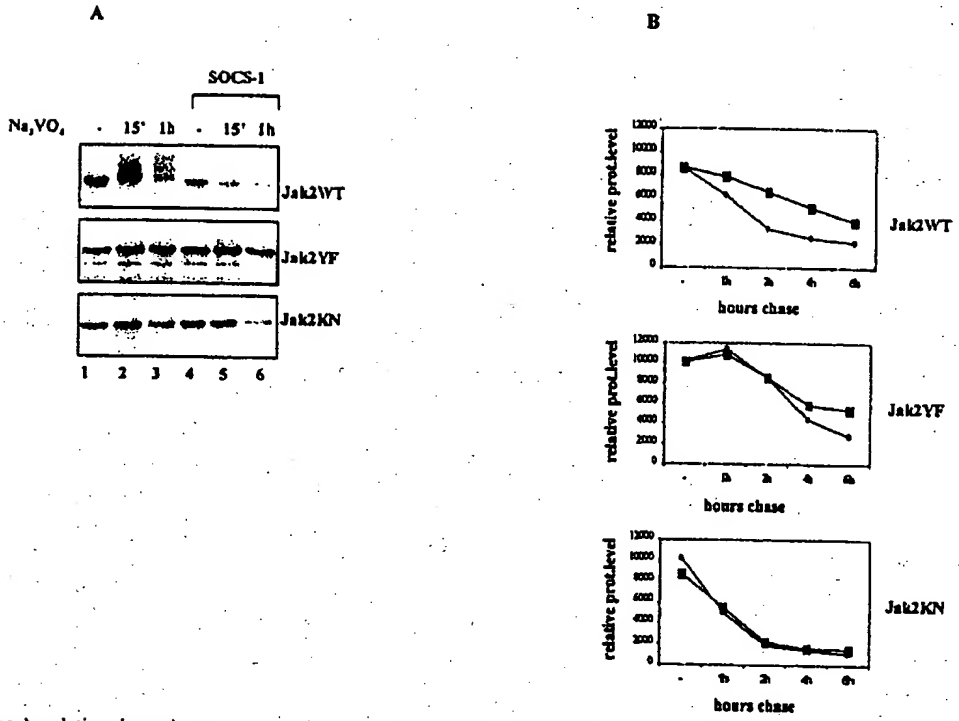

FIG. 7. Phosphorylation-dependent proteasomal degradation of Jak2WT is enhanced by SOCS-1 coexpression. (A) SOCS-1 coexpression enhances the degradation of Jak2WT but not Jak2YF or Jak2KN in vivo. Cos-7 cells were transfected with Jak2 plasmid (3 μg) either alone or with the SOCS-1 plasmid (5 μg) and pretreated with proteasome inhibitor MG132 (20 μM) for 1 h before ^{35}S metabolic labeling. The inhibitor was maintained throughout the pulse-labeling experiment. Cells were then treated with pervanadate (25 μM) and lysed in NP-40 lysis buffer supplemented with 25 μM MG132. Jak2 protein levels were analyzed by immunoprecipitation using an anti-HA antibody followed by autoradiographic detection. (B) Half-lives of different Jak2 mutants in the absence (square) or presence (circle) of SOCS-1 coexpression. Cos-7 cells were transfected with different Jak2 plasmids alone or together with the SOCS-1 plasmid and pulse-labeled with ^{35}S for 15 min and chased for the indicated periods. Jak2 protein (prot) levels were analyzed by anti-HA immunoprecipitation followed by autoradiographic exposure. Images were scanned and quantified by using the Quan-Image5 program, and the relative protein amounts were adjusted so that time zero represents 100.

nation of Jak2, we analyzed the effects of SOCS-1 and SOCS-1ΔSB lacking the SOCS box (58) in an in vitro assay (55). Jak2WT-HA, Jak2YF-HA, and Jak2KN-HA were immunopurified from Cos-7 cells and incubated in the presence of SOCS-1, SOCS-1ΔSB, or control lysates. The ubiquitination reaction was allowed to proceed for 1 h, and the ubiquitination of Jak2 was analyzed by Western blotting. The results (Fig. 9) show that SOCS-1 induced ubiquitination of Jak2WT, whereas SOCS-1ΔSB had no significant effect. SOCS-1 did not affect the ubiquitination of Jak2YF and Jak2KN mutants. These results suggest that SOCS-1 induces polyubiquitination of Jak2WT, which requires the presence of the SOCS box of SOCS-1 and phosphorylation of Y1007 on Jak2.

DISCUSSION

Appropriate biological responses through cytokine receptors require stringent control of the activation signals. The importance of negative regulation is well exemplified by the results obtained from deregulated and aberrantly activated Jak tyrosine kinases that cause autonomous cell growth and cancer (26, 33). Here we show that the ubiquitin-proteasome pathway directly regulates tyrosine-phosphorylated Jak2 in cytokine receptor signaling.

The irreversible nature of the proteasomal degradation of proteins demands strict control for these processes. The specificity is mainly determined through a large family of E3 ubiquitin ligases, which recognize substrate proteins in highly or-

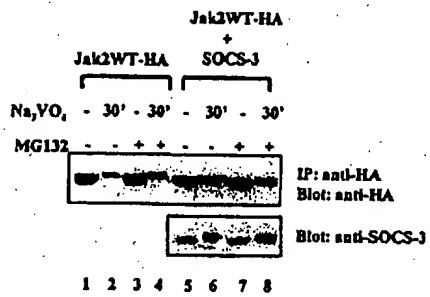

FIG. 8. SOCS-3 does not affect Jak2 protein levels in Cos-7 cells. Cos-7 cells were transiently transfected with Jak2WT (4 μg) or with SOCS-3 (4 μg) plasmids. After 48 h the cells were starved overnight and treated with MG132 and pervanadate as described for Fig. 5. Jak2 was analyzed by immunoprecipitation (IP) and immunoblotting using an anti-HA antibody. SOCS-3 protein levels were determined by Western blotting 20 μg of total-cell lysates using an anti-SOCS-3 antibody.

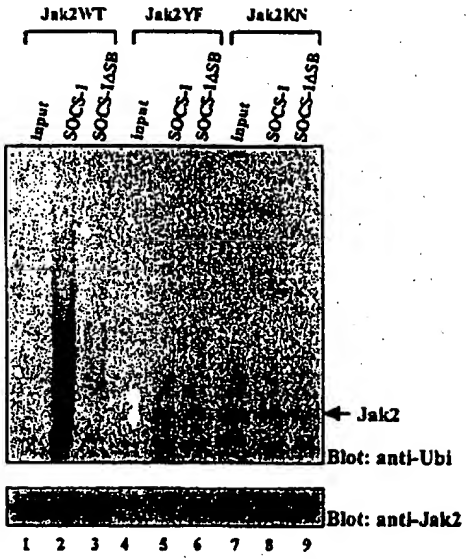

FIG. 9. SOCS-1 enhances the ubiquitination of Jak2WT in vitro. Immunopurified Jak2WT, Jak2YF, and Jak2KN were subjected to an in vitro ubiquitination reaction in the presence SOCS-1, SOCS-1ΔSB, or control lysates. The ubiquitination of Jak2 was analyzed after 1 h of in vitro reaction by antiubiquitin Western blotting. The filter was stripped and reblotted with an anti-Jak2 antibody.

dered protein complexes (6). Protein phosphorylation has been shown to function as a regulatory mechanism for ubiquitination and proteasomal degradation of various signaling proteins. Several cell surface receptors as well as cellular and transcriptional regulators such as cyclins, cyclin-dependent kinase inhibitors, Src kinases, IκB-α, p53, β-catenin, c-Jun, and RNA polymerase have been shown to be regulated via phosphorylation-dependent proteasomal degradation (27). Tyrosine phosphorylation was also a critical regulator of ubiquitination and degradation of Jak2. Tyrosine phosphorylation stimulated polyubiquitination of Jak2, which was stabilized by proteasome inhibitor treatment, thus indicating that polyubiquitinated Jak2 is rapidly degraded through the proteasome pathway. We did not observe any significant downregulation in Jak2 protein levels in 32D cells after IL-3 stimulation. Based on our experience, only a small fraction of cellular Jak2 becomes activated and tyrosine phoshorylated after IL-3 stimulation, and the degradation of this fraction is likely to be difficult to detect on the total-protein level. The relatively short half-life of unstimulated Jak2 is also consistent with the concept that the turnover of Jak2 functions as a regulatory mechanism in cytokine signaling (43).

A likely mechanism for the tyrosine phosphorylation-dependent regulation of ubiquitination is specific recruitment of the ubiquitination enzyme complex to the protein. The catalytic activation of Jak2 involves auto- or transphosphorylation of the activation loop Y1007, which is likely to cause a conformational change that allows substrates and ATP to access the catalytic pocket (12). Here we show that proteasomal degradation of Jak2 required phosphorylation of Jak2 on Y1007. The phosphorylated Y1007 on Jak2 serves also as a docking site for the extended SH2 domain of SOCS-1, but an additional 12 N-terminal amino acids, constituting what is termed the kinase-inhibitory region, confer high-affinity binding to the kinase domain and inhibitory interaction with the catalytic pocket of Jak2 (52). The interaction of the SOCS box with elongins B and C suggested that the regulation via SOCS proteins may involve the ubiquitin-proteasome pathway (57). Recent results from mice with a deletion of the SOCS box of SOCS-1 demonstrate a clear physiological regulatory function for the SOCS box (58). These mice have a phenotype similar to but less severe than that of SOCS-$1^{-/-}$ mice and show increased IFN-γ responsiveness and develop a lethal inflammatory disease. We showed that SOCS-1 enhanced ubiquitination of Jak2 in a SOCS box-dependent manner and that this effect was regulated by Y1007 on Jak2. Thus SOCS-1 is likely to recruit, along with elongins, the E3 ligase to the phosphorylated Jak2. The exact composition of the SOCS-1–elongin B/C ubiquitination complex is currently unknown but it is likely to resemble that of the von Hippel-Lindau complex and to consist of RING finger protein Rbx1 as a bridging factor, Cullin-2, and an E2-conjugating enzyme. In cotransfection experiments SOCS-1 also induced Jak2 degradation which was dependent on Y1007. SOCS-1 has also been shown to induce degradation of the VAV protein through the ubiquitin-proteasome pathway (9). We did not observe any significant reduction in total Jak2 protein levels in IFN-γ-treated thymocytes from SOCS-$1^{-/-}$ mice (data not shown). However, there was a decrease in antiubiquitin-reactive high-molecular-weight protein complexes, which are typically observed with polyubiquitinated proteins. These results are consistent with the concept that ubiquitination of Jak2 is reduced in SOCS-$1^{-/-}$ thymocytes, but more-detailed studies are required to resolve this issue. Interestingly, SOCS-3, which associates weakly with Jak2 (42), did not have any effect on Jak2 protein levels. These findings support the concept that different SOCS proteins have highly specialized functions in regulation of cytokine receptor signaling.

Proteasome inhibitors stabilized the Jak2–SOCS-1 complex and inhibited proteolysis of Jak2. It is of interest that SOCS-1 protein levels were not markedly affected by pervanadate treatment. We could not detect significant levels of ubiquitination of SOCS-1 (data not shown); thus, while SOCS-1 may act as part of an E3 ubiquitin ligase complex, it is not a substrate of the E3 ligase. These findings together with results from mice with a SOCS box deletion are consistent with a sequential-regulation model for SOCS-1, which is initiated by an inhibitory interaction with activated Jak2, followed by recruitment of the E3 ubiquitin ligase complex and resulting in ubiquitination and subsequent targeting of Jak2 to proteasomes for degradation. In line with this hypothesis, cytokine stimulation has been shown to enhance the interaction between SOCS-1 and elongins B and C, possibly due to conformational changes induced by SOCS-1–Jak2 association (57).

Jak2 was found to be monoubiquitinated to various degrees in different cells. This finding was somewhat surprising, but monoubiquitination of Jak2 was observed by using three different lysis conditions as well as an HA-tagged ubiquitin expression vector in Cos-7 cells. Monoubiquitination of Jak2 did not appear to be strictly tyrosine phosphorylation dependent, as both Jak2KN in Cos-7 cells and endogenous Jak2 in unstimulated cells were monoubiquitinated. Induction of tyrosine

phosphorylation, however, stimulated both monoubiquitination and polyubiquitination of Jak2. Attachment of a single ubiquitin molecule on a target substrate (i.e., monoubiquitination) either modulates the activity or localization of the protein or targets the protein to the proteasomes (6, 17). Intriguingly, monoubiquitination of proteins may increase the efficiency of the polyubiquitination reaction (31). For Jak2, monoubiquitination may "sensitize" the activated kinase to the subsequent SOCS-1-mediated polyubiquitination, ensuring that activated Jak2 is degraded in a timely fashion. The regulatory mechanisms for the monoubiquitination of Jak2 are currently unknown, and it remains possible that the monoubiquitination reaction involves an E3 ubiquitin ligase complex different from the one which catalyzes the polyubiquitination of the tyrosine-phosphorylated Jak2.

Proteasome inhibitors stabilized the tyrosine-phosphorylated form of Jak2, which suggests that the physiological function of the ubiquitin-proteasome pathway is to remove the active tyrosine kinase and prevent aberrant cytokine signaling. Recently SOCS-1 was shown to mediate the growth inhibition of TEL-Jak2-transformed cells and to accelerate the proteasomal degradation of the oncogenic, hyperactive TEL-Jak2 and gyrase B-Jak2 chimeras, neither of which is activated in response to cytokines (13, 20). These results are in accordance with our results showing that IL-3 and IFN-γ stimulations and tyrosine phosphorylation of Jak2 on Y1007 regulate ubiquitination and degradation of the cellular Jak2. Regulation of hyperactivated and oncogenic forms of Src kinases through ubiquitin-mediated degradation has also been shown; thus proteasomal degradation may be a common mechanism to control the activity of tyrosine kinases (15, 55). In agreement with this hypothesis, mutations that inhibit the ubiquitin ligase activity of RING finger protein c-Cbl are oncogenic and cause failure to ubiquitinate and desensitize EGFR (28). The regulatory mechanisms involved in these processes have to possess high degrees of specificity, and E3 ligase E6AP appears to be responsible for ubiquitination of Src kinases, and c-Cbl mediates the ubiquitination of EGFR (15, 38), while the SOCS-1-recruited ubiquitination complex represents one mechanism involved in regulation of Jak2.

The SOCS-1-mediated ubiquitination and degradation of Jak2 show some analogy to the c-Cbl-mediated desensitization of EGFR (28). The phosphorylated Y1045 on EGFR serves as a docking site for RING finger protein c-Cbl, which regulates the ubiquitination of the receptor. Phosphorylation of c-Cbl on Y371 flanking its RING finger domain is critical for the ubiquitination process and subsequent degradation of the activated EGFR. The Jak2–SOCS-1 interaction does not involve direct tyrosine phosphorylation of SOCS-1 (data not shown), but whether the function of SOCS-1 is regulated by other post-translational modifications or protein interactions remains to be investigated. In this regard it is interesting that cytokine stimulation has been shown to promote the interaction between SOCSs and elongins B and C (57).

It is becoming evident that the ubiquitin-proteasome pathway plays an integral part in regulation of cytokine receptor signaling. Several cytokine receptors were shown to be ubiquitinated upon ligand stimulation, and their turnover and intracellular sorting are regulated through proteasomes (49, 54). SOCS family protein Cis has been implicated in proteasome-mediated regulation of EpoR (49). Here we show that ubiquitination plays a critical role in selecting the activated Jak2 for degradation. Interaction between SOCS-1 and tyrosine-phosphorylated Jak2 stimulates proteasomal degradation of Jak2, which may regulate, e.g., IFN-γ and granulocyte-macrophage colony-stimulating factor signaling (32). Various SOCS proteins display highly specific functions in regulation of individual cytokine signaling pathways, and it is likely that other SOCSs or distinct adapter proteins and ubiquitination enzyme complexes regulate other cytokine signaling pathways. Finally, proteasome inhibitors stabilize transcriptionally active tyrosine-phosphorylated STAT5 via an ubiquitin-independent mechanism, which represents a distinct mechanism of proteasomal regulation (51). In summary, regulation of cytokine receptor signaling cascades that activate Jak2 and STAT transcription factors involves several highly specific ubiquitin-proteasome-dependent regulatory mechanisms, and the specificity of this regulation is determined by interactions with ancillary proteins such as SOCSs.

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The SOCS Box of SOCS-1 Accelerates Ubiquitin-dependent Proteolysis of TEL-JAK2*

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Fusion of the *TEL* gene on 12p13 to the JAK2 tyrosine kinase gene on 9p24 has been found in human leukemia. *TEL*-mediated oligomerization of JAK2 results in constitutive activation of the tyrosine kinase (JH1) domain and confers cytokine-independent proliferation on interleukin-3-dependent Ba/F3 cells. Forced expression of the JAK inhibitor gene SOCS1/JAB/SSI-1 induced apoptosis of TEL-JAK2-transformed Ba/F3 cells. This suppression of TEL-JAK2 activity was dependent on SOCS box-mediated proteasomal degradation of TEL-JAK2 rather than on kinase inhibition. Degradation of JAK2 depended on its phosphorylation and its high affinity binding with SOCS1 through the kinase inhibitory region and the SH2 domain. It has been demonstrated that von Hippel-Lindau disease (VHL) tumor-suppressor gene product possesses the SOCS box that forms a complex with Elongin B and C and Cullin-2, and it functions as a ubiquitin ligase. The SOCS box of SOCS1/JAB has also been shown to interact with Elongins; however, ubiquitin ligase activity has not been demonstrated. We found that the SOCS box interacted with Cullin-2 and promoted ubiquitination of TEL-JAK2. Furthermore, overexpression of dominant negative Cullin-2 suppressed SOCS1-dependent TEL-JAK2 degradation. Our study demonstrates the substrate-specific E3 ubiquitin-ligase-like activity of SOCS1 for activated JAK2 and may provide a novel strategy for the suppression of oncogenic tyrosine kinases.

Cytokines induce the activation of the JAK family tyrosine kinases (JAKs)[1] and the subsequent recruitment of various signaling proteins to the receptor complex, including the STAT family of transcription factors. Constitutive activation of the JAK/STAT pathway has been found in many leukemic cell lines, including cells transformed with Bcr-Abl (1, 2), as well as in human T-cell lymphotrophic virus-1-transformed T cells (3, 4). A constitutively activated form of STAT5 conferred factor-independent growth on Ba/F3 cells (5), and that of STAT3 has also been shown to function as an oncogene (6). Moreover, a constitutively activated JAK kinase generated by chromosome translocation between the *TEL* gene on 12p13 and the JAK2 gene on 9p24 has been shown to be associated with human leukemia (7, 8). TEL, a subset of the ETS family of transcription factors, contains a conserved oligomerization domain, known as the PNT domain, in the N-terminal region. Like other TEL-tyrosine kinase fusion proteins such as the TEL-PDGF receptor β chain and TEL-Abl, the JAK2 tyrosine kinase domain is constitutively activated by oligomerization mediated by the PNT domain. Stable expression of TEL-JAK2 confers factor-independent growth on IL-3-dependent Ba/F3 cells and induces myeloproliferative and T-cell lymphoproliferative diseases in mice (9).

The JAK/STAT pathway is regulated by several mechanisms, including dephosphorylation by protein phosphatases and degradation by the ubiquitin/proteasome system (see review; Yasukawa *et al.*(10)). The CIS family (also referred to as the SOCS or SSI family) has been shown to play an important role in regulating cytokine signal transduction. CIS1, the first member of this family to be cloned, suppresses STAT5 activation by binding to cytokine receptors (11, 12). The second family member found, JAB/SOCS1/SSI-1, directly binds to the JAK2 kinase (JH1) domain, thereby inhibiting tyrosine kinase activity (13–15). Mutational analysis and biochemical characterization revealed a novel type of inhibition of JAK2 tyrosine kinase activity through the two independent binding sites of SOCS1/JAB: the N-terminal kinase inhibitory region binds to the catalytic groove of JH1, and the SH2 domain binds to the phosphorylated tyrosine residue Tyr-1007 in the activation loop (16, 17). Gene disruption studies have suggested that one of the major physiological functions of SOCS1 is the negative regulation of the IFNγ/STAT1 pathway (18, 19).

Six additional CIS/SOCS/SSI family members were cloned from a data base search (20–22). In this family, the SH2 domain and the C-terminal region of about 40 amino acids, referred to as the SOCS box, are conserved. The data base search also revealed that a similar SOCS box is present in several proteins containing ankyrin-like repeats, Ras-like GTPases, or WD40 domains (20, 22). The SOCS box has been implicated in protein stability or degradation of associated molecules, because it was found to interact with the Elongin B and Elongin

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[1] The abbreviations used are: JAK, JAK family tyrosine kinases; STAT, signal transducers and activators of transcription; PDGF, platelet-derived growth factor; IL-3, interleukin-3; IFNγ, interferon γ; Cul-2, Cullin-2; VHL, von Hippel-Lindau disease; Elongin B,C, Elongin B and C complex; PCR, polymerase chain reaction; HA, hemagglutinin; WT, wild type; dC40, deletion mutant lacking 40 amino acids at the C terminus; GST, glutathione *S*-transferase; EPOR, erythropoietin receptor; EGFP, expressed green fluorescence protein.

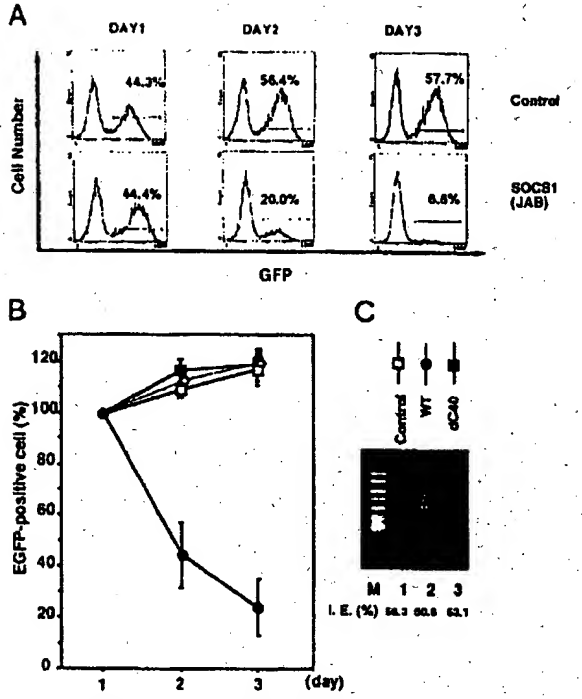

FIG. 1. **Suppression of TEL-JAK2-dependent growth of Ba/F3 cells by SOCS1.** *A*, BF/TEL-JAK cells were infected with retrovirus carrying IRES-EGFP alone (*Control*) or wild-type SOCS1. The numbers of EGFP-positive cells were scored by flow cytometry on the indicated day after infection. Cells were cultured in the absence of IL-3. *B* and *C*, BF/TEL-JAK cells were infected with a control virus (*open square* and *lane 1*) or a retrovirus carrying wild-type-SOCS1 (*WT*) (*closed circle* and *lane 2*), or a dC40 mutant SOCS1 (*dC40*) (*closed square* and *lane 3*). BF/Bcr-Abl cells were also infected with a retrovirus carrying WT-SOCS1 (*open circle*). The mean and standard error shown are from three independent experiments. *C*, a DNA fragmentation assay was performed 24 h after infection. Infection efficiency (*I.E.*), which is the percentage of EGFP-positive cells, is shown at the *bottom*. *M*, DNA size marker.

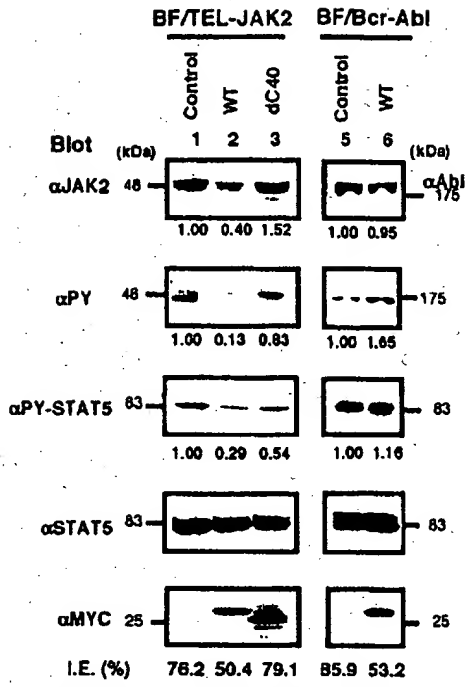

FIG. 2. **Expression levels of TEL-JAK2 after wild-type and mutant SOCS1 infection.** BF/TEL-JAK or BF/Bcr-Abl cells were infected with retrovirus carrying the indicated cDNAs. One day after infection, infection efficiency (*I.E.*) was determined as the EGFP-positive fraction. Then the cells were lysed and immunoblotted with the indicated antibodies. The phosphorylated forms of TEL-JAK2 and Bcr-Abl detected with anti-PY (*αPY*) antibody were determined from their molecular size. The intensity of the bands was quantified by a densitometer and normalized with that of control virus-infected cells.

C (Elongin B,C) complex, which may recruit Cullin-2 (Cul-2), Rbx1, and the E2 ubiquitin-conjugating enzyme (23–25). Therefore, CIS family members are hypothesized to function as E3-like ubiquitin-ligase complexes against target molecules from an analogy with the von Hippel-Lindau (VHL) tumor-suppressor gene product. However, no evidence in support of this hypothesis has yet been reported. Kamura *et al.* (23) reported that the protein levels of full-length JAK2 were not affected by coexpression of SOCS1, and, rather, that coexpression of Elongin B,C stabilized the SOCS1 protein. Furthermore, it has been shown that the SOCS box is not essential for the inhibition of cytokine-induced JAK/STAT activation by SOCS1 (16, 17, 23, 26). Therefore, the role of the SOCS box of SOCS1 still remains to be elucidated.

To suppress the oncogenic potential of activated tyrosine kinases, we introduced the SOCS1 gene into Ba/F3 cells transformed with TEL-JAK2 using a retrovirus system. Overexpression of SOCS1 could efficiently suppress the transforming potential of TEL-JAK2. However, simple inhibition of kinase activity by SOCS1 could not explain the suppression of TEL-JAK2. We found that SOCS1 promoted ubiquitin-proteasome-dependent degradation of TEL-JAK2 and full-length JAK2, and that this process required the C-terminal SOCS box of SOCS1 as well as the phosphorylation of JAK2.

EXPERIMENTAL PROCEDURES

Cells and Transfection—Murine IL-3-dependent Ba/F3 cells were maintained in RPMI medium supplemented with 10% fetal calf serum and 10% conditioned medium from WEHI-3B cells as a source of IL-3. Ba/F3 cells were transformed with pCDNA3-TEL-JAK2 or pCDNA3-Bcr-Abl as described previously (11). After selection with G418 (1 mg/ml), cells that could grow without IL-3 were subsequently selected. Transient transfection and the luciferase assay in 293 cells have been described previously (20).

cDNA Construction—Deletion, substitution, and chimeric mutants were generated by standard PCR methods as described previously (16, 27). Some of the mutants and wild-type SOCS1 were subcloned into a pMX-IRES-EGFP vector (28). For swapping of the SOCS box, the SOCS boxes of SOCS1 (codon 167–212), CIS3/SOCS3 (codon 180–225), and CIS1 (codon 213–257) were interchanged by introducing an *SpiI* site at the joint. All constructs contained an N-terminal Myc- or FLAG-tag (13). For TEL-JAK2 fusion, the human TEL (codon 1–162) part was obtained by PCR and fused to the mouse JAK2 JH1 domain (codon 839–1127). This fusion gene corresponds to the TEL-JAK2 found in B-cell lymphoblastic leukemia patients (9). Murine Cul-2 cDNA was obtained by PCR from a brain cDNA library and cloned into a pCDNA3 vector containing an N-terminal HA-tag. The R452C mutant was created by site-directed mutagenesis.

Retrovirus Production and Infection—Retroviruses were produced by transient transfection of the PLAT-E packaging cell line with cDNAs in pMX-IRES-EGFP (28). Forty-eight hours after transfection, the culture supernatant was harvested and stored at −80 °C. BF/TEL-JAK or BF/Bcr-Abl cells (2×10^5 cells) were infected with appropriately diluted PLAT-E supernatants containing 10 μg/ml Polybrene for 24 h. After being washed, cells were further cultured in an RPMI medium for an additional 24 or 48 h. Then, aliquots of cells (1×10^4) were analyzed using a Coulter EPICS-XL flow cytometer. All experiments were performed in the absence of IL-3.

In Vitro Kinase Assay—An *in vitro* kinase assay for TEL-JAK2 was performed as described previously (16). Briefly, FLAG-tagged TEL-

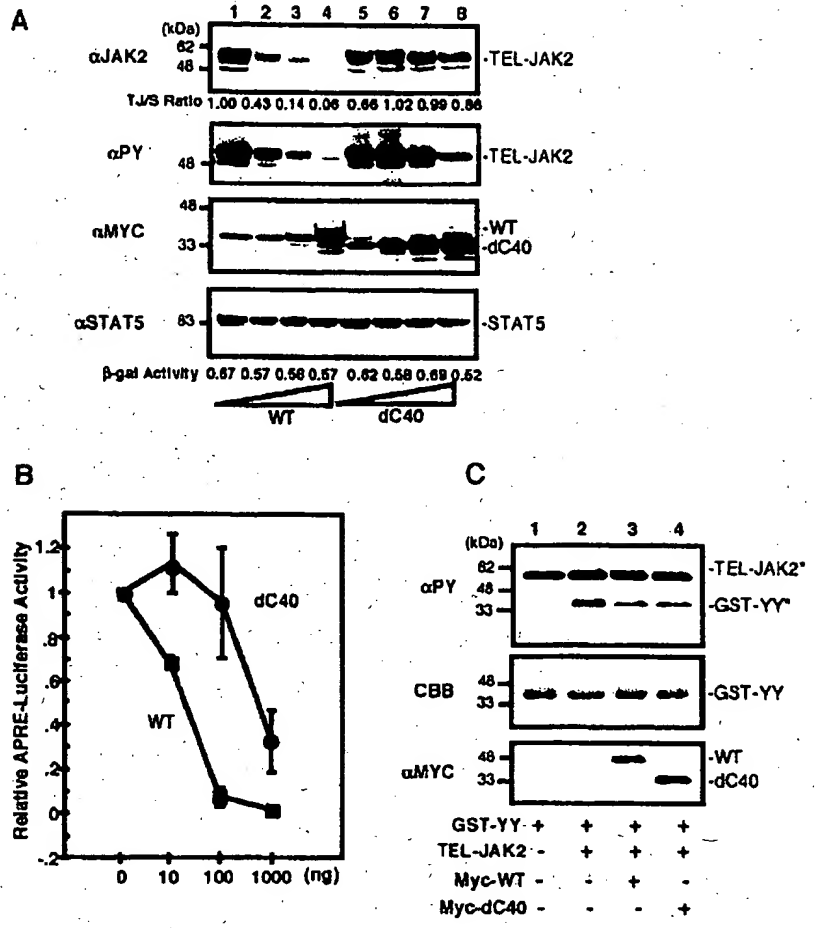

FIG. 3. **Effect of WT and dC40-SOCS1 on TEL-JAK2 stability in 293 cells and *in vitro* kinase activity.** 293 cells were transfected with pCDNA3 carrying TEL-JAK2 (0.1 μg), pCDNA3 carrying Myc-tagged WT-SOCS1 (*WT*) or dC40-SOCS1 (*dC40*) (0.001, 0.01, 0.1, and 1.0 μg), β-galactosidase (0.1 μg), and the APRE reporter gene (0.5 μg) that can monitor STAT3 and STAT5 activity. The cell lysate (30 μg of protein/lane) was prepared and subjected to immunoblotting (*A*) with the indicated antibodies and a luciferase assay (*B*). The values of β-galactosidase activity as an indicator of transfection efficiency are listed in *A*. The membrane was reprobed with anti-STAT5 (*αSTAT5*) to show equal loading of the samples, and the intensity of the bands was quantified by a densitometer. The relative ratio (*TJ/S ratio*) of the band intensity of TEL-JAK2 *versus* that of STAT5 is shown in *A*. In *C*, FLAG-tagged TEL-JAK2 expressed in 293 cells was purified by anti-FLAG antibody-conjugated protein G-Sepharose. After incubation with 293 cell lysates containing WT- or dC40-SOCS1 for 1 h at 4 °C, the beads were reacted with GST-YY as a substrate in the presence of 50 μM ATP for 5 min. The reaction mixture was separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with αPY and αMyc antibodies. GST-YY was stained with Coomassie Brilliant Blue (*CBB*).

JAK2 expressed in 293 cells (3.0 μg/transfection) grown in 10-cm dishes was immunoprecipitated with anti-FLAG antibody in 60 μl (50% v/v) of protein G-Sepharose. Then the resin was incubated with 1 ml of cell extracts from 293 cells transiently expressing wild-type SOCS1 (WT) or a deletion mutant lacking 40 amino acids at the C terminus (dC40) at 4 °C for 1 h. After being washed twice with kinase reaction buffer (50 mM Hepes-buffer, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 μM dithiothreitol, and 10 μM Na_3VO_4), the beads were resuspended in 20 μl of kinase reaction buffer containing the substrate polypeptide, GST-EPOR cytoplasmic domain (GST-YY) (16) (0.1 mg/ml), and ATP (50 μM) and incubated at 30 °C for 5 min. Kinase activity was analyzed by immunoblotting of GST-YY with anti-phosphotyrosine (4G10).

Immunoprecipitation and Western Blot Analysis—Immunoprecipitation and immunoblotting were performed as described previously (11). Anti-JAK2 JH1 (αJAK2) rabbit polyclonal antibody, anti-Myc (αMYC) monoclonal and polyclonal antibodies, and anti-phosphotyrosine (αPY, 4G10) antibodies have been described previously (16). For pulse-chase experiments, 293 cells (1×10^6) grown in 10-cm dishes were transfected with TEL-JAK2 (2.0 μg of plasmid) and WT or dC40 cDNA (0.02 μg). After 18 h, the cells were pulse-labeled with Tran^{35}S-label (ICN) at a concentration of 150 μCi/ml for 15 min and then scraped. After being divided into four parts, cells were replated into 3.5-cm dishes. Following the indicated chase periods, cells were lysed and immunoprecipitated with anti-JAK2 antibody followed by protein A-Sepharose, separated on SDS-polyacrylamide gel electrophoresis, exposed, and quantified by a BAS-2000 imaging system (Fuji). To see the effect of proteasome inhibitors, 293 cells transfected with TEL-JAK2 and WT-SOCS1 were treated with lactacystin or MG132 (25 μM each) for 30 min before labeling. The drugs were maintained throughout the pulse-chase period. For the cycloheximide treatment experiment, 293 cells (1×10^6) were transfected with 2.0 μg of TEL-JAK2 plus WT or mutant SOCS1 (0.02 μg). Eighteen hours after transfection, cells were trypsinized and divided into four parts. After a 5-h incubation period, cells were attached to the dishes and then treated with 50 μg/ml cycloheximide for the indicated periods. The cell extracts were prepared and immunoblotted with anti-JAK2, anti-STAT5, and anti-Myc antibodies as described (16). Band intensity was quantified by a densitometer as described (16).

RESULTS

SOCS Box-dependent Suppression of TEL-JAK2 Transforming Activity by SOCS1—To determine whether the JAK inhibitor SOCS1/JAB can suppress oncogenic tyrosine kinases, SOCS1 cDNA was introduced into Ba/F3 cells transformed with either TEL-JAK2 (BF/TEL-JAK) or p210 Bcr-Abl (BF/Bcr-Abl) together with enhanced green fluorescence protein (EGFP) using the bicistronic retrovirus vector pMX-IRES-EGFP (28). Because the infected cells expressed both EGFP and Myc-tagged SOCS1, the percentage of infected cells was determined as the EGFP-positive rate by flow cytometry. The same virus was shown to induce apoptosis of parental Ba/F3 cells in the presence of IL-3 (28). As shown in Fig. 1 (*A* and *B*), the population of wild-type SOCS1 (WT)-infected BF/TEL-JAK cells decreased markedly, suggesting that WT-infected cells disappeared with cell death. Indeed, WT-infected BF/TEL-JAK cells underwent apoptosis characterized by DNA fragmentation (Fig. 1*C*, *lane 2*). Similar effects were observed even in the presence of IL-3 (data not shown). WT did not affect the growth of BF/Bcr-Abl cells (Fig. 1*B*), which is consistent with the observation that SOCS1 did not inhibit Bcr-Abl tyrosine kinase

activity (data not shown). Thus, the inhibitory effect of SOCS1 was shown to be specific to JAKs.

We have shown that the N-terminal kinase inhibitory region and the SH2 domain, but not the C-terminal SOCS box, are essential for SOCS1 to inhibit JAK kinase activity *in vitro* and *in vivo* (16). However, unexpectedly, the dC40 mutant lacking the entire SOCS box could not suppress the growth of BF/TEL-JAK cells (Fig. 1, *B* and *C*). To clarify the reason for this discrepancy, we examined STAT5 tyrosine phosphorylation in BF/TEL-JAK cells after infection (Fig. 2; infection efficiency is listed as *I.E.*). Infection with WT and dC40, but not with a control virus, resulted in a decrease in the tyrosine phosphorylation of STAT5 (Fig. 2, *αPY-STAT5*). Reduction of STAT5 phosphorylation by WT was more profound than that by dC40. Tyrosine phosphorylation of TEL-JAK2 was partially reduced by dC40, suggesting that dC40 could reduce TEL-JAK2 kinase activity but was not sufficient to induce apoptosis of BF/TEL-JAK cells at the expression levels obtained by the retrovirus system. It should be noted that the infection efficiency of the WT virus to BF/TEL-JAK2 judged by flow cytometry was less than 55%, whereas those of dC40 and control viruses were more than 75%, even though virus titers were similar when assayed with NIH-3T3 cells. This is presumably because WT virus-infected cells die rapidly after infection. Thus, the infection efficiency of the WT virus will be underestimated. More drastically, we noticed that the protein levels of TEL-JAK2 decreased in WT-infected cells but not in dC40-infected cells (Fig. 2, *αJAK2*). WT-SOCS1 did not affect the protein level of Bcr-Abl (Fig. 2, *αAbl*). We found a 70–80% decrease of TEL-JAK2 and PY-STAT5 levels in WT virus-infected cells, whereas the infection efficiency was only 50%. This is probably because of the underestimation of the infection efficiency of WT-infected cells. These data suggest that kinase inhibition by SOCS1 is not sufficient to suppress the oncogenic potential of TEL-JAK2 and that the C-terminal SOCS box is necessary for complete suppression of the oncogenic potential of TEL-JAK2 by reducing the TEL-JAK2 protein level.

SOCS1 Promotes Proteasome-dependent Degradation of TEL-JAK2—We clarified the mechanism of reduction of the protein level of TEL-JAK2 by coexpression of SOCS1 using a transient expression system in 293 cells. As shown in Fig. 3*A*, WT, but not dC40-SOCS1, also reduced the level of TEL-JAK2 in a dose-dependent manner in 293 cells (Fig. 3*A*, *αJAK2*). Thus, SOCS1 reduced the TEL-JAK2 protein level SOCS-box-dependently not only in Ba/F3 cells but also in 293 cells. Consequently, WT suppressed the TEL-JAK2-mediated STAT activation ~50 times more efficiently than dC40 (Fig. 3*B*). To confirm a similar kinase inhibitory activity of WT and dC40, we performed an *in vitro* kinase assay using the recombinant protein of the GST-tagged erythropoietin receptor (EPOR) cytoplasmic domain as substrate (16). Consistently, with the previous study using GST-JH1 as a constitutively activated kinase, WT and dC40 could similarly suppress the *in vitro* kinase activity of TEL-JAK2 (Fig. 3*C*, *lanes 3* and *4*). These results indicate that, although the SOCS box of SOCS1 is not necessary for kinase inhibition, the inhibitory effect of WT-SOCS1 was strongly enhanced by inducing degradation of TEL-JAK2 (Fig. 3, *A* and *B*).

The half-life of TEL-JAK2 was examined in a metabolic pulse labeling and chase experiment (Fig. 4*A*). The half-life of TEL-JAK2 was over 60 min, but coexpression of WT accelerated the decay of TEL-JAK2, reducing its half-life to less than 30 min. dC40 did not affect the half-life of TEL-JAK2. Accelerated degradation of TEL-JAK2 in the presence of WT, but not dC40, was also observed after cells were treated with a protein synthesis inhibitor, cycloheximide (Fig. 4*B*). Normalized levels of

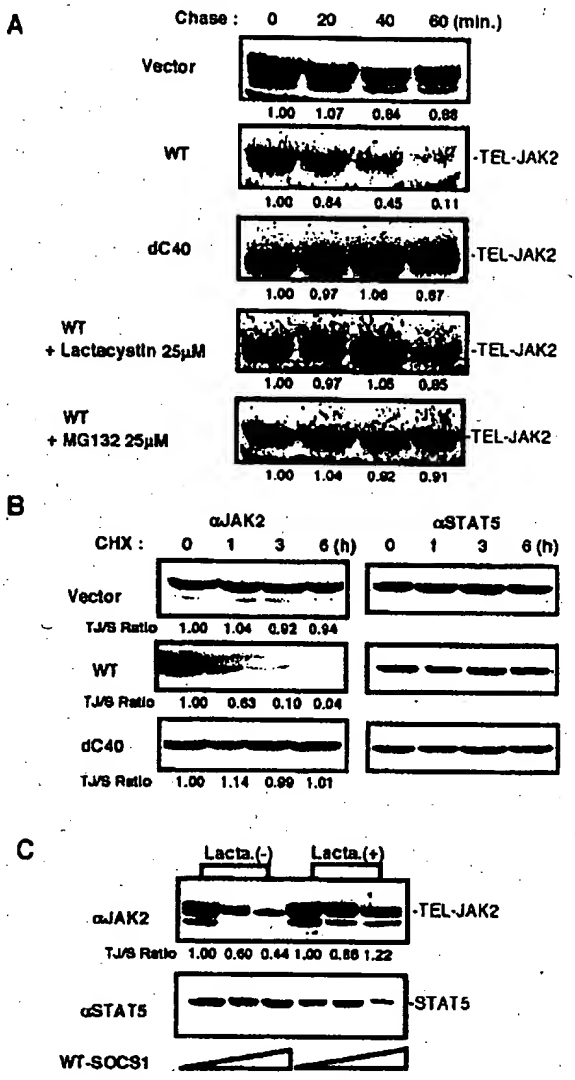

FIG. 4. **The SOCS box-dependent proteasomal degradation of TEL-JAK2 by the coexpression of SOCS1.** *A*, pulse-chase experiment. 293 cells transfected with TEL-JAK2 together with control vector (*Vector*), WT-SOCS1 (*WT*), or dC40-SOCS1 (*dC40*) were pulse-labeled with [^{35}S]methionine and cysteine for 15 min, divided into four parts, and then chased for indicated periods. The TEL-JAK2 protein was immunoprecipitated with anti-JAK2. For proteasome inhibitor treatment, 25 μM lactacystin (*Lacta.*) or MG132 was included in the medium throughout the pulse-chase periods. The relative intensity of the bands quantified by a densitometer is shown. *B*, cycloheximide treatment experiment. 293 cells were transfected with TEL-JAK2 together with control vector (*Vector*), WT, or dC40. After 18 h, cells were divided into four parts and incubated for an additional 5 h. Then, the cells were incubated with 50 μg/ml cycloheximide (*CHX*) for the indicated periods and immunoblotted with αJAK2 and αSTAT5 antibodies. The TJ/S ratio (see Fig. 3*A* legend) is listed. *C*, 293 cells transfected with TEL-JAK2 (0.1 μg) and WT-SOCS1 (*lanes 1*, *4*, 0 μg; *lanes 2*, *5*, 0.01 μg; *lanes 3*, *6*, 0.1 μg) were treated without (*lanes 1–3*) or with 25 μM lactacystin (*lanes 4–6*) for 6 h and then immunoblotted with αJAK2 and αSTAT5 antibodies.

TEL-JAK2 are shown as the TJ/S ratio against the levels of STAT5, which is a very stable protein (Fig. 4*B*).

As shown in Fig. 4 (*A* and *C*), the rapid degradation of TEL-JAK2 by coexpression of SOCS1 was significantly delayed by treatment of the cells with two proteasome inhibitors, lac-

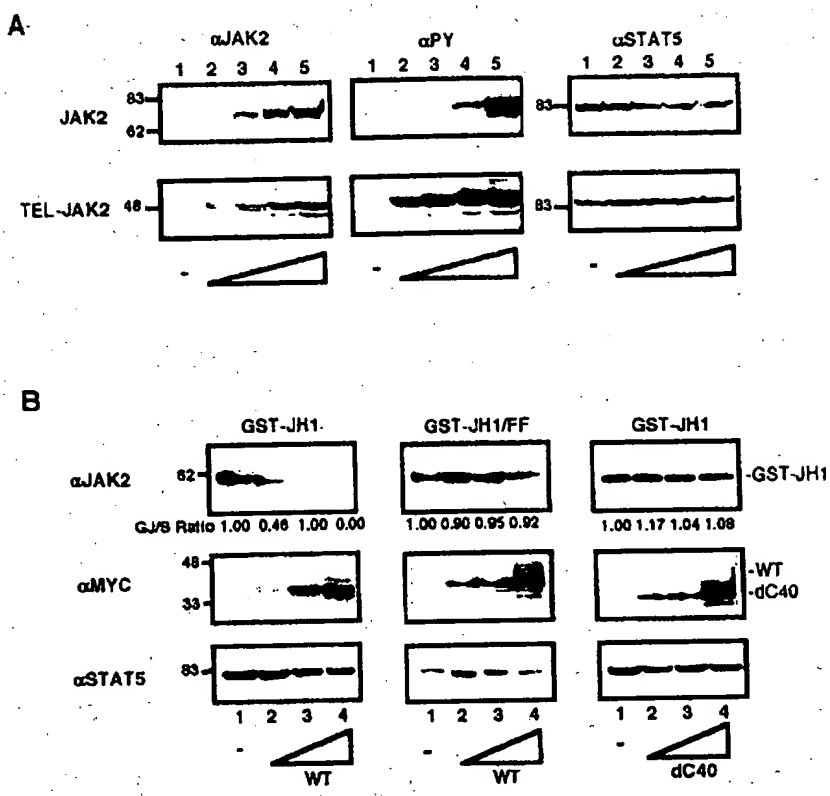

FIG. 5. **Phosphorylation of the JH1 domain is required for SOCS1-induced degradation.** *A*, 293 cells were transfected with various concentrations of plasmids encoding either full-length JAK2 (*upper panel*) or TEL-JAK2 (*lower panel*). The cell lysate was immunoblotted with the indicated antibodies. Plasmid concentrations are 0, 0.03, 0.1, 0.3, and 1.0 μg from *lanes 1* to *5*. *B*, 293 cells were transfected with 0.1 μg of GST-JH1 or GST-JH1/FF plasmids together with increasing concentrations of WT-SOCS1 or dC40-SOCS1 (0, 0.01, 0.1, and 1.0 μg). The cell lysate was immunoblotted with the indicated antibodies. The relative ratio (*GJ/S ratio*) of the band intensity of GST-JH1 *versus* that of STAT5 is shown.

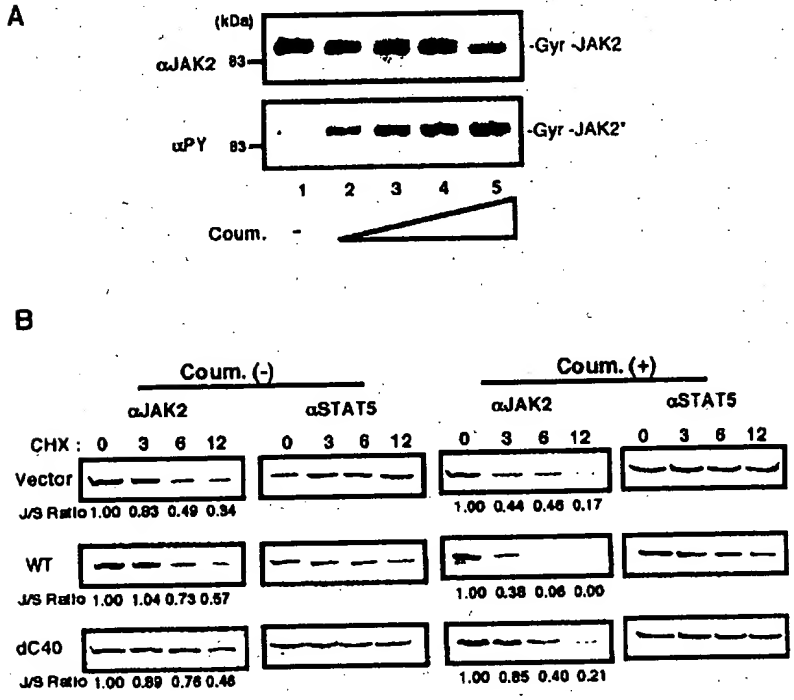

FIG. 6. **SOCS1 accelerated degradation of full-length JAK2 by forced dimerization.** *A*, 293 cells transfected with 0.1 μg of Gyrase-JAK2 fusion gene (*Gyr-JAK2*) were treated with 0, 0.01, 0.1, 1, and 10 μM coumermycin for 1 h. The cell lysates were immunoprecipitated with αJAK2, then immunoblotted with αJAK2 or αPY antibodies. The *asterisk* indicates the phosphorylated form. *B*, 293 cells transfected with 0.1 μg of Gyr-JAK2 together with 0.1 μg of pCDNA3 (*Vector*), WT-SOCS1 (*WT*), and dC40-SOCS1 (*dC40*) were incubated with (+) or without (−) 1 μM coumermycin in the presence of 50 μg/ml cycloheximide for the indicated periods. Cell lysates were immunoblotted with αJAK2 and αSTAT5 antibodies. The relative ratio (*J/S ratio*) of the band intensity of Gyr-JAK2 *versus* that of STAT5 is shown.

tacystin and MG132. These data suggest that SOCS1 promotes proteasome-dependent degradation of TEL-JAK2.

Phosphorylation of the JH1 Domain Is Necessary for SOCS1-mediated Degradation of JAK2—Previously, Kamura *et al.* (20) reported that SOCS1 overexpression did not affect wild-type JAK2 protein stability. We tried to resolve this discrepancy

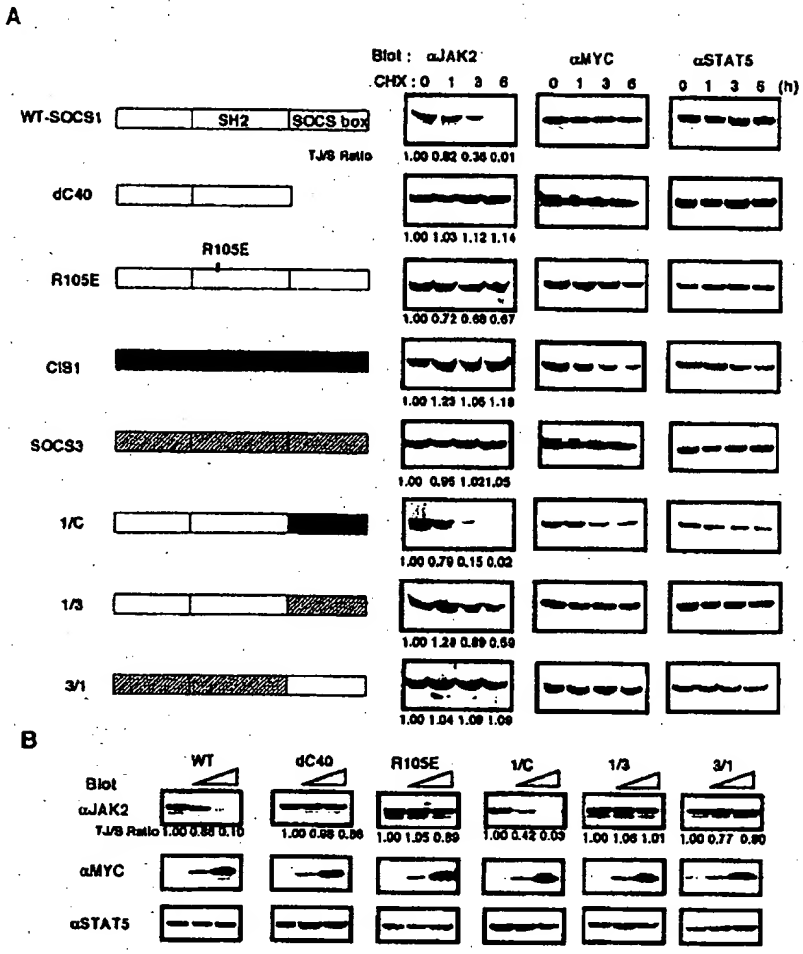

FIG. 7. **The effect of SOCS box swapping on TEL-JAK2 degradation.** *A*, schematic structures of SOCS1, CIS1, and SOCS3 and their chimeric constructs are shown on the *left*. 293 cells transfected with TEL-JAK2 and each construct were incubated with 50 μg/ml cycloheximide for the indicated periods and immunoblotted with αJAK2, αSTAT5, and αMYC antibodies. The TJ/S ratio is listed. *B*, TEL-JAK2 (0.1 μg) and WT or indicated mutant SOCS constructs (0, 0.01, or 0.1 μg of plasmids) were transfected into 293 cells. Total cell lysates were blotted with the indicated antibodies.

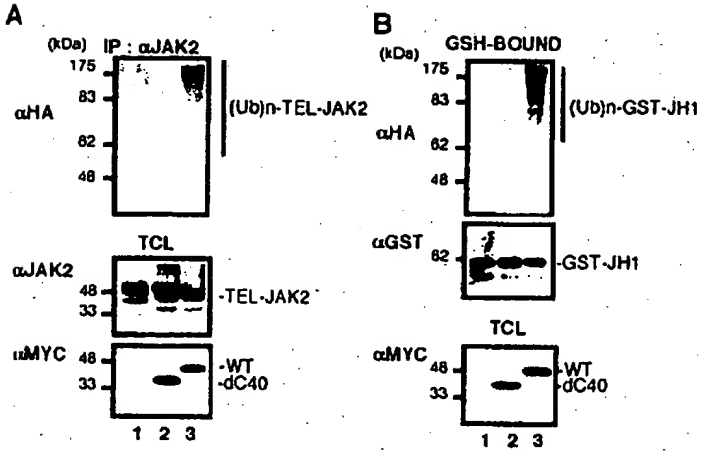

FIG. 8. **SOCS1 promotes the ubiquitination of activated JAK2.** 293 cells were transfected with 1 μg TEL-JAK2 (*A*) or GST-JH1 (*B*) and 0.5 μg of HA-ubiquitin/pCDNA3 together with a 0.01-μg control vector (*lane 1*) or plasmids carrying dC40 (*lane 2*) or WT-SOCS1 (*lane 3*). To keep the levels of TEL-JAK2, relatively low amounts of SOCS1 plasmid (0.01 μg) were used. The cell extracts were precipitated with αJAK2 (*A*) or GSH-Sepharose (*B*) and then blotted with anti-HA (*αHA*) and αGST antibodies. Total cell lysates (*TCL*) were also blotted with the indicated antibodies.

between TEL-JAK2 and full-length JAK2. As shown in Fig. 5*A*, full-length JAK2 was much less tyrosine-phosphorylated than TEL-JAK2 when expressed alone in 293 cells. Therefore, we suspect that the phosphorylation of the JH1 domain is necessary for SOCS1-mediated degradation. Because glutathione *S*-transferase (GST) is a dimer, the JH1 domain fused to GST (GST-JH1) is another constitutively activated form of the JAK2 tyrosine kinase domain (16). Like TEL-JAK2, GST-JH1 was markedly decreased in its expression level when coexpressed with WT, but not with dC40 (Fig. 5*B*). Pulse-chase experiments revealed that WT-SOCS1 also shortened the half-life of GST-JH1 (data not shown). Moreover, the protein level of the phosphorylation-deficient mutant (FF) of GST-JH1 was not affected by SOCS1 (Fig. 5*B*). Therefore, reduction in the GST-JH1 pro-

tein level by SOCS1 was dependent on tyrosine phosphorylation or the activation of the JH1 domain.

Next, we examined whether SOCS1 promotes the degradation of activated full-length JAK2. To achieve the activation of JAK2, JAK2 was fused to gyrase B (Gyr-JAK2), and Gyr-JAK2 was dimerized by coumermycin (29). As shown in Fig. 6*A*, coumermycin treatment enhanced the tyrosine phosphorylation of Gyr-JAK2. Without coumermycin, WT-SOCS1 did not affect the protein levels of JAK2 (Fig. 6*B*, *left*). However, WT, but not dC40, induced the degradation of Gyr-JAK2 in the presence of coumermycin (Fig. 6*B*, *Coum. (+)*, *αJAK2*). These data suggest that the SOCS1-SOCS box can potentially induce the degradation of full-length JAK2 but that this process requires tyrosine phosphorylation (or activation) of JAK2.

The SOCS Box of SOCS1 Can Be Replaced with That of CIS1 but Not That of SOCS3—To examine the functional redundancy of the SH2 domain and the SOCS box for TEL-JAK2 degradation, we constructed chimeric mutants among CIS1, CIS3/SOCS3, and SOCS1 (Fig. 7). The SH2 domain mutant R105E-SOCS1 exhibited a lesser effect on TEL-JAK2 protein stability. Thus, tight binding of SOCS1 to the JH1 domain through the SH2 domain is necessary for the degradation of TEL-JAK2. CIS1, which does not bind to JAK2, did not induce TEL-JAK2 degradation, although CIS1 itself was unstable compared with SOCS1 and SOCS3 (see αMYC blot). SOCS3, which also suppresses JAK2 signaling (27), did not induce the degradation of TEL-JAK2. As shown in Fig. 7 (*A* and *B*), the mutant SOCS1 whose SOCS box was replaced with that of CIS1 (*1/C*) reduced the TEL-JAK2 level, whereas the mutant replaced with the SOCS box of SOCS3 (*1/3*) did not. 1/C induced the degradation of TEL-JAK2 more strongly than did WT-SOCS1. This indicates that the SOCS box of SOCS1 can be replaced with that of CIS1 but not with that of SOCS3. On the other hand, the SOCS3 mutant whose SOCS box was replaced with that of SOCS1 (*3/1*) did not affect TEL-JAK2 stability. SOCS3 and 3/1 bound to the JH1 domain, but their affinity was much lower than that of WT-SOCS1 or 1/C (27). All these observations were confirmed when different amounts of wild-type or mutant SOCS/CIS genes were expressed (Fig. 7*B*). These data suggest that the particular SOCS box and its tight binding or proper orientation to the JH1 domain are necessary for the promotion of the degradation of TEL-JAK2.

SOCS1-SOCS Box Promotes the Ubiquitination of TEL-JAK2, and Dominant Negative Cul-2 Inhibits TEL-JAK2 Degradation—Because proteasome inhibitors suppressed SOCS1-mediated degradation of TEL-JAK2, ubiquitination of TEL-JAK2 may be involved in the degradation process. The SOCS box is similar to the BC box of VHL protein, which interacts with the Elongin B,C complex. The VHL-Elongin B,C (VBC) complex further recruits Cul-2 and Rbx-1 as subunits of ubiquitin ligase. Rbx-1 has a RING-finger motif with which E2 ubiquitin-conjugating enzymes are suggested to interact. Kamura *et al.* (23) and Zhang *et al.* (24) demonstrated that the SOCS1-SOCS box binds to the Elongin B,C complex. Therefore, the SOCS box has been hypothesized to form a complex with Cul-2 and Rbx-1 and function as an E3 ubiquitin ligase (25). However, neither the existence of this complex nor the SOCS box-dependent ubiquitination of the target molecule has been demonstrated to date.

First, we examined the ubiquitination of TEL-JAK2 and GST-JH1 using HA-ubiquitin. As shown in Fig. 8 (*A* and *B*), the ubiquitination of TEL-JAK2 and GST-JH1 was markedly enhanced when they were coexpressed with WT-SOCS1, whereas the dC40 mutant did not induce their ubiquitination. Next, we examined the interaction between the SOCS box and Cul-2 (Fig. 9*A*). HA-tagged Cul-2 was coprecipitated with WT, but not

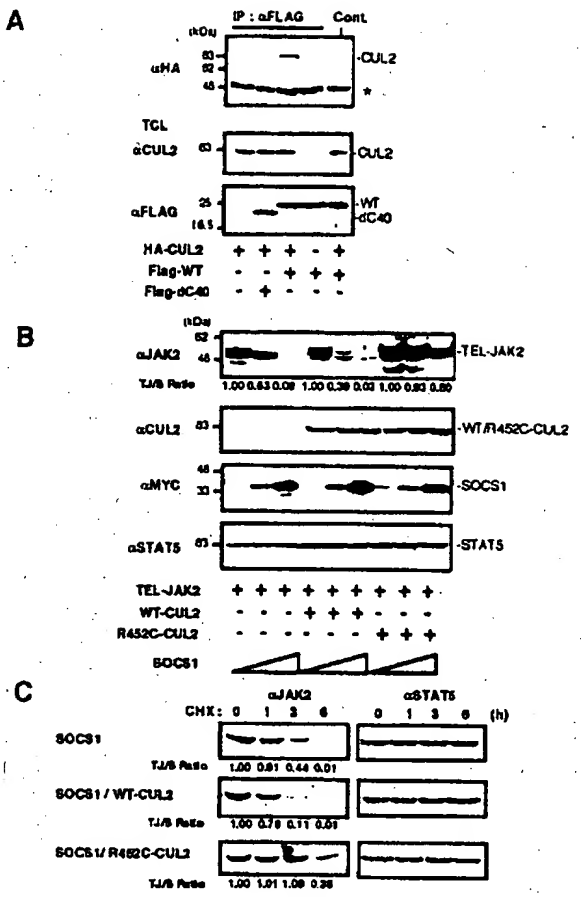

FIG. 9. **A dominant negative Cul-2 inhibits the SOCS1-mediated degradation of TEL-JAK2.** *A*, 293 cells were transfected with HA-Cul-2 and FLAG-tagged WT-SOCS1 or dC40-SOCS1 and then immunoprecipitated with anti-FLAG (*αFLAG*) or control (unrelated monoclonal) antibodies. The immunoprecipitates or total cell lysates were blotted with the indicated antibodies. The *asterisk* shows a nonspecific IgG band. *B*, 293 cells were transfected with TEL-JAK2 and either wild-type Cul-2 (*WT-CUL2*) or R452C mutant Cul-2 (*R452C-CUL2*) (0.5 μg) together with 0.01, 0.1, or 1.0 μg of WT-SOCS1 plasmids. The cell lysates were immunoblotted with the indicated antibodies. The TJ/S ratio is listed. *C*, 293 cells transfected with TEL-JAK2 and WT-SOCS1, together with either WT-Cul-2 or R452C mutant Cul-2, were incubated with 50 μg/ml cycloheximide for the indicated periods and immunoblotted with αJAK2 or αSTAT5 antibodies. The TJ/S ratio is listed.

with dC40, in 293 cells. We also confirmed the binding of the Elongin B,C complex with WT-SOCS1 (data not shown). Thus, similarly to VHL, the SOCS1-SOCS box can interact with the Elongin B,C complex and Cul-2. Overexpression of wild-type Cul-2 (*WT-CUL2*) accelerated SOCS1-induced TEL-JAK2 degradation (Fig. 9, *B* and *C*), whereas SOCS1-induced degradation of TEL-JAK2 was almost completely blocked when mutant Cul-2 containing R452C substitution (*R452C-CUL2*) was coexpressed (Fig. 9, *B* and *C*). R452 of Cul-2 corresponds to Arg-488 of Cdc53, and mutation of this residue may disrupt the interaction with Rbx1 (30, 31). Thus, R452C-Cul-2 functioned as a dominant negative form of wild-type Cul-2. Taken together, these observations suggest that the functional recruitment of Elongin B,C and the Cul-2 complex through the SOCS box accelerated the ubiquitination and proteasome-dependent degradation of TEL-JAK2.

DISCUSSION

It has been demonstrated that the SOCS boxes of VHL and SOCS1 interact with the Elongin B,C complex. Although the VHL-SOCS box is proposed to recruit Cul-2 and Rbx-1 and function as an E3 enzyme subunit, the function of the SOCS box of the CIS/SOCS family has not been resolved. In this paper, we demonstrate that the SOCS box of SOCS1 is critically involved in the ubiquitin/proteasome-dependent degradation of TEL-JAK2 and phosphorylated JAK2.

By analogy with the VHL complex, the SOCS boxes of SOCS1 and CIS1 were suggested to be involved in the ubiquitin/proteasome-dependent degradation of JAK2 and the EPO receptor, respectively (25, 32). Indeed, CIS1 itself is shown to be ubiquitinated and degraded very rapidly (32). Because the tyrosine phosphorylation of the EPO receptor in response to EPO was sustained by the treatment of cells with proteasome inhibitors, we proposed the possibility that the phosphorylated EPO receptor-CIS1 complex becomes a target of the proteasome. Zhang *et al.* (24) also suggested that the SOCS box of SOCS3 leads the SOCS3 protein to the degradation pathway, because SOCS3 degradation was blocked by proteasome inhibitors. In this case, the SOCS box may be involved in ubiquitination of SOCS3 itself. However, there is no direct evidence that SOCS box-containing proteins have an E3-like ubiquitin-transfer activity with the target molecule. Kamura *et al.* (23) reported that the Elongin B,C complex increased the stability of SOCS1 and that SOCS1 overexpression did not induce the degradation of JAK2.

It is notable that SOCS3 did not affect TEL-JAK2 degradation, because SOCS3 has a SOCS box and can bind to JH1. The SOCS3-SOCS box was not functional when fused to the SOCS1 N-terminal region and the SH2 domain (*1/3* mutant in Fig. 7). We found that SOCS3 lacking a SOCS box is much more stable than wild-type SOCS3 in Ba/F3 cells (data not shown). This suggests that the SOCS box of SOCS3 may not regulate the protein levels of the target molecule but rather destabilize SOCS3 itself. Like CIS1, SOCS3 has been shown to be an unstable molecule that is degraded by the ubiquitin/proteasome system (24). Therefore, the SOCS box may be involved in inter- as well as intramolecular ubiquitin transfer, depending on the structure of the SOCS box. Our experiments do not exclude the possibility that SOCS-3 has another target besides JAKs or cytokine receptors.

Using constitutively active forms of JAK2, we demonstrated that the SOCS1-SOCS box could indeed induce ubiquitination and proteasome-dependent degradation of JAK2. Our study indicated that the SOCS-box-dependent degradation of JAK2 requires the phosphorylation of JAK2 and strong interaction with SOCS1. Confirming the result of Kamura *et al.* (23), SOCS1 did not induce the degradation of full-length JAK2 when these two molecules were simply expressed in 293 cells. This was probably due to the low efficiency of the phosphorylation of JAK2 molecules (see Fig. 5*A*). Similarly to TEL-JAK2, phosphorylated full-length JAK2 by forced dimerization was degraded by coexpression of SOCS1. Thus, acceleration of the degradation of SOCS1 is dependent on the activation (or phosphorylation) of JAK2.

It is still not clear whether this accelerated degradation of full-length JAK2 occurs under physiological conditions. Callus and Mathey-Prevot (33) reported that proteasome-dependent degradation is the major mechanism of the down-regulation of JAK2 after stimulation with IL-3 in Ba/F3 cells. SOCS1 or other CIS/SOCS members may be involved in this process. However, we and other researchers have reported that the SOCS box of SOCS1 has no apparent role in the suppression of cytokine-dependent signaling in 293 cells (16, 17). In these assays, SOCS1 was overexpressed by transient transfection before cytokine stimulation. We tried to see the effect of SOCS1 on the degradation of activated JAK2 in response to IFNγ using embryonic fibroblasts from wild-type and SOCS1−/− mice. We could confirm that IFNγ-induced JAK2 phosphorylation was prolonged in SOCS1−/− fibroblasts; however, we did not detect any decrease in the protein level of JAK2 in either cell types. This is probably because only a small fraction of JAK2 is phosphorylated in response to IFNγ. Further studies are necessary to address the role of the SOCS box on JAK ubiquitination and degradation in physiological conditions.

After completion of this study, De Sepulveda *et al.* (34) reported that SOCS-1 accelerated the ubiquitination and degradation of Vav. Vav and SOCS1 form a protein complex through interactions between the Vav N-terminal regulatory region and the SH2 domain of SOCS1 in a phosphotyrosine-independent manner. Thus, SOCS1 may induce the degradation of Vav when expressed at very high levels. It has not been reported whether or not the SOCS box of SOCS1 is necessary for the ubiquitination of Vav. Therefore, the molecular mechanism of the ubiquitination of Vav by SOCS1 is still unclear. Moreover, it has not been demonstrated that such phosphorylation-independent interaction can occur in physiological conditions. However, it would be interesting to determine whether SOCS1 can induce ubiquitination and degradation of phosphorylated signaling molecules other than JAKs. In future studies, it will be possible to verify the physiological role of the SOCS box of SOCS1 by introducing mutations in the SOCS box using a knock-in strategy in mice.

SOCS-box-mediated ubiquitination and degradation of activated JAK2 are reminiscent of c-Cbl-mediated ubiquitination and degradation of the activated receptor tyrosine kinases (35–37). Other groups as well as ours have shown that the c-Cbl RING-finger domain interacts with the E2 ubiquitin-conjugating enzyme, thereby accelerating the ubiquitination of the epidermal growth factor receptor or the PDGF receptor with which c-Cbl binds through its SH2 domain. Thus, ubiquitination and proteasome-dependent degradation of activated tyrosine kinases by a specific E3 complex may be a common mechanism in the down-regulation of tyrosine kinases. In addition, we have reported that c-Cbl suppressed EPO-induced STAT5 activation in collaboration with APS (an adaptor containing PH and SH2 domains) (38). Because Cbl family members possess the SH2 domain, multiple tyrosine phosphorylation sites, and proline-rich motifs that interact with SH3 domains, they can interact with many tyrosine-phosphorylated proteins as well as SH2- and SH3-containing proteins. Therefore, Cbl could also be involved in ubiquitin/proteasome-dependent degradation of activated cytokine receptors and their downstream signal transducers. Our study also provides a basis for the inhibition of oncogenic, constitutively active tyrosine kinases by ubiquitination and degradation using the SOCS box or the RING-finger domain.

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Accelerated Publication

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Suppressor of Cytokine Signaling-1 Inhibits VAV Function through Protein Degradation*

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Suppressor of cytokine signaling-1 (SOCS1) is an inducible Src homology 2 (SH2)-containing protein that negatively regulates cytokine and growth factor signaling required during thymic development. Recent evidence indicates that SOCS1 interacts with elongins B and C, which are components of a ubiquitin ligase complex, VCB (VHL/elonginC/B), based on the VHL (von Hippel Lindau) tumor suppressor protein. SOCS1 has previously been shown to operate as an inhibitor of Janus kinases. Here we show that SOCS1 has the distinct function of targeting the hematopoietic specific guanine nucleotide exchange factor, VAV, for ubiquitin-mediated protein degradation. VAV and SOCS1 form a protein complex through interactions between the VAV NH_2-terminal regulatory region and the SH2 domain of SOCS1 in a phosphotyrosine-independent manner. SOCS1 decreases the steady state levels of cotransfected VAV and onco-VAV and reduces the focus forming activity of onco-VAV. SOCS1 stimulates the polyubiquitination of VAV proteins *in vivo*, which was stabilized by proteasomal inhibitors. These results suggest that SOCS1 programs VAV degradation by acting as a substrate-specific recognition component of a VCB-like ubiquitin ligase complex.

The suppressor of cytokine signaling (SOCS)[1] family is composed of eight related SH2-containing proteins and represents a class of adapter molecules that negatively regulate diverse cytokine signaling pathways (1–4). SOCS1 is expressed in the thymus and cells of hematopoietic origin (1, 5). SOCS1 is part of an autoregulatory loop in which SOCS1 induction, following cytokine or growth factor receptor stimulation, attenuates Janus kinase (JAK) activity (1–3, 5). Consistent with this model, targeted disruption of the SOCS1 locus in mice gives rise to a syndrome of perinatal lethality and thymic atrophy resulting from unbridled interferon-γ signaling (6–8). SOCS1 inhibits JAK by binding, via its SH2 domain, to the positive regulatory tyrosine in the kinase domain activation helix (2, 9). Although SOCS1 also binds to the receptor tyrosine kinases Kit and Flt3, it does not suppress the kinase activity of these receptors (5). Nevertheless, SOCS1 potently blocks KIT- and FLT3-induced proliferation, suggesting that SOCS1 may modulate signaling through a mechanism distinct from kinase inhibition (5).

In addition to its interaction with receptor and non-receptor tyrosine kinases, SOCS1 binds to the hematopoietic-specific guanine nucleotide exchange factor, VAV (5). VAV contains several modular protein domains including an NH_2-terminal calponin homology (CH), an acidic region, a Dbl-homology (DH) domain, a pleckstrin homology (PH) domain, a cysteine-rich region, and two SH3 domains flanking an SH2 domain (reviewed in Ref. 10 and references therein). VAV converts inactive Rac-GDP into active Rac-GTP, which in turn regulates cytoskeletal reorganization and the activation of the c-*jun* NH_2-terminal kinase (JNK) and p38HOG. VAV exchange factor activity is stimulated by tyrosine phosphorylation following antigen and cytokine receptor activation. VAV activity is also enhanced by its relocalization to the plasma membrane through interaction of its pleckstrin homology domain with phosphatidylinositol 3,4,5-trisphosphate. Deletion of the VAV NH_2-terminal sequences renders VAV oncogenic (11). VAV is negatively regulated by its NH_2-terminal sequences (12) through an unknown mechanism. Here, we provide evidence that SOCS1 binds to the NH_2 terminus of VAV and stimulates its ubiquitin-dependent degradation.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Antibodies—NIH 3T3, COS7, and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 5% fetal bovine serum (Hyclone). Plasmid pCMV-VAV was constructed by inserting murine VAV cDNA in the vector pFlag-CMV-6b (Kodak). pEF-VAV and pEF-onco-VAV were gifts from Dr. A. Altman (San Diego). pMT3-SOCS1 expresses hemagglutinin (HA) epitope-tagged murine SOCS1 from the CMV promoter (5). A point mutation, R105K, within the SH2 domain of SOCS1 abolishes its ability to bind phosphotyrosine residues (5). pEF-SOCS1 was provided by T. Kishimoto (13). pEF-FlagCis, pEF-FlagSOCS2, and pEF-FlagSOCS3 were from D. Hilton (14). RasV12 was expressed from the plasmid pT22 (15). The HA-ubiquitin construct in pMT123 was from D. Bohmann (16). HA-SOCS1 was detected using either a rabbit polyclonal anti-serum raised against SOCS1 (aa 1–172) (5) or anti-HA monoclonal antibody 12CA5 as indicated in the figures. Rabbit polyclonal anti-VAV antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag monoclonal antibody M2 was from Sigma.

Transfection and Foci Formation Assays—293T cells were transfected by the calcium phosphate method (5). COS7 cells were trans-

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[1] The abbreviations used are: SOCS1, suppressor of cytokine signaling; HA, hemagglutinin; VHL, von Hippel Lindau protein; VCB, VHL-elongin B-elongin C complex; SH2 and SH3, Src homology 2 and 3, respectively; JAK, Janus kinase; JNK, c-*jun* NH_2-terminal kinase; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; aa, amino acid; GST, glutathione *S*-transferase.

fected with Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. After 36–48 h, cells were lysed and subjected to immunoprecipitation and Western blotting as described earlier (5). For foci forming assays, 1.5×10^5 NIH 3T3 cells in 100-mm dishes were transfected 24 h later by calcium phosphate method with plasmids expressing onco-VAV in the presence or absence of SOCS1 along with 5 μg of mouse genomic DNA as carrier. The cells were fixed 10–14 days post-transfection in 10% formaldehyde for 10 min and stained overnight with 0.02% Giemsa stain.

Pulse-Chase Experiments—COS7 cells transfected with VAV or onco-VAV in the presence or absence of SOCS1 were washed 36–48 h after transfection and incubated in Cys- and Met-free DMEM (ICN). After 30 min, the medium was replaced with Cys, Met- free DMEM containing 2.5% dialysed calf serum and 100 μCi/ml Trans^{35}S-label™ (ICN, Specific activity 1175 Ci/mmol) to label the newly synthesized proteins. Following 20 min of labeling, the dishes were washed once in phosphate-buffered saline and incubated in chase medium (DMEM containing 10% fetal calf serum and 10-fold molar excess of Cys and Met). At the end of the indicated chase period, the cells were washed and lysed. The immunoprecipitated VAV or onco-VAV proteins, separated by SDS-PAGE, were detected by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In Vivo Ubiquitination of VAV—*In vivo* ubiquitination was performed essentially as described by Marti *et al.* (17). COS7 cells were cotransfected with plasmids expressing VAV, HA-SOCS1, and HA-ubiquitin. After 36–48 h, the cells were washed, lysed in SDS lysis buffer (50 mM Tris, pH 7.4, 1% SDS, and 1 mM dithiothreitol), boiled, and diluted 10-fold before immunoprecipitation with anti-VAV antibody. Proteasomal inhibitor epoxomycin (Affinity, Exeter, UK) was added during the final 8 h before lysis. An aliquot of the diluted lysate was used to monitor the ubiquitination of total cellular proteins and the expression of SOCS1. Ubiquitinated VAV was detected with anti-HA antibody.

RESULTS

SOCS1 Binds to the NH_2 Terminus of VAV—To identify proteins that interact with SOCS1, we used the full-length murine SOCS1 cDNA as a bait to screen a cDNA library in the yeast two-hybrid system and isolated a clone encoding the NH_2-terminal region of VAV (5). To determine which region of SOCS1 is involved in this interaction, the NH_2 terminus of SOCS1 (aa 1–79), the central SH2 domain (aa 64–172) or the COOH terminus (aa 168–212) were expressed as LexA fusion proteins along with VP16-VAV in yeast. The isolated SOCS1 SH2 domain bound to VAV at levels similar to that of full-length SOCS1, whereas neither the NH_2- nor the COOH-terminal regions of SOCS1 were sufficient to mediate VAV binding (not shown). To confirm the VAV-SOCS1 interaction in mammalian cells, HA-tagged SOCS1 was cotransfected with full-length murine VAV cDNA into 293T cells, and coimmunoprecipitation was analyzed by Western blotting. VAV was detected in SOCS1 immune complexes derived from cells transfected with both cDNAs but not from cells transfected with VAV or HA-SOCS1 alone (Fig. 1*A*). Similarly, SOCS1 was detected in VAV immunoprecipitates (data not shown). We then tested whether SOCS1 could bind to onco-VAV, an activated form of VAV that lacks the first 67 residues of the wild type protein (11). SOCS1 coprecipitated onco-VAV, indicating that the NH_2-terminal sequences deleted in onco-VAV are not required for the SOCS1 interaction. To determine whether the VAV acidic domain was sufficient for SOCS1 binding, GST fusion proteins containing the entire NH_2 terminus or the minimal acidic domain (aa 116–199) were expressed and used as affinity reagents. Both constructs were capable of binding to SOCS1 from cell lysates, whereas the entire NH_2-terminal polypeptide (aa 1–199) failed to bind to a control protein (Fig. 1*C*). These results were recapitulated using the yeast two-hybrid assay in which the acidic domain of VAV (aa 116–199) interacted with SOCS1 (data not shown).

The interaction of VAV and SOCS1 in yeast suggested that the interaction did not require phosphotyrosine modification as has been reported for other SH2-containing proteins (18, 19).

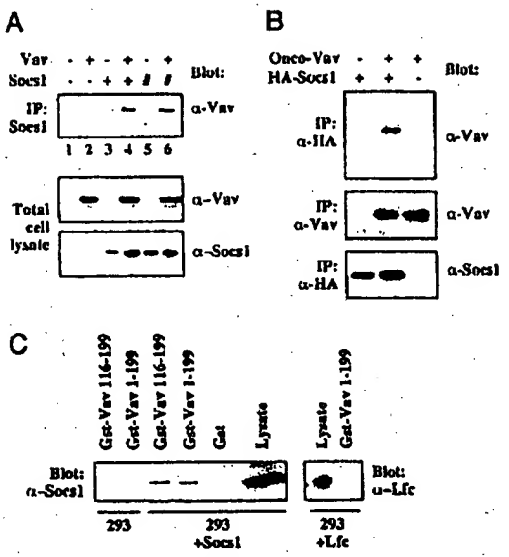

FIG. 1. **SOCS1 interacts with VAV and onco-VAV in a P-Tyr independent fashion.** 293T cells were transfected with plasmids expressing VAV (*A*) or onco-VAV (*B*) with SOCS1 or empty vector. SOCS1 immunoprecipitated with anti-SOCS1 or anti-HA was probed for coprecipitated VAV and onco-VAV by Western blot. In *lanes 5* and *6* (*A*), a loss-of-function mutant of SOCS1 (R105K) was used. *Bottom panels* show the levels of VAV and SOCS1 in whole cell lysates or immunoprecipitates (*IP*) as indicated. *C*, SOCS1 binds to the acidic region of VAV. Bacterially expressed GST-VAV fusion proteins encoding VAV amino acids 1–199 or 116–199 were incubated with lysates from untransfected 293T cells, 293T cells transfected with SOCS1, or a control protein, Lfc. The GST fusion proteins recovered on a glutathione-Sepharose affinity matrix were probed for SOCS1 or Lfc using specific antibodies.

Consistent with this finding, VAV was coprecipitated with a mutant SOCS1 (Fig. 1*A*, *lane 6*) that is unable to bind to tyrosine phosphorylated proteins as a result of R105K susbstitution in the phosphotyrosine-binding pocket of the SH2 domain (5). These data demonstrated that SOCS1 SH2 binds to the acidic region of VAV in a phosphotyrosine-independent fashion.

SOCS1 Suppresses VAV-induced Focus Formation—To determine a functional interaction between SOCS1 and VAV proteins, the effect of SOCS1 was tested on the foci forming ability of onco-VAV in NIH 3T3 cells (11). Cotransfection of SOCS1 reduced foci formation by 50-fold (Fig. 2), whereas SOCS3, the most closely related SOCS family member, did not alter the number of foci. Consistent with the fact that the R105K mutation in SOCS1 did not affect VAV binding, the oncogenic potential of onco-VAV was inhibited by the cotransfected SOCS1-R105K. The effect of SOCS1 on VAV was specific, since SOCS1 did not attenuate the transformation induced by the activated Ras protein RasV12 (Fig. 2).

SOCS1 Destabilizes VAV Protein—During the course of these experiments, we observed that the steady state levels of transfected VAV and onco-VAV were diminished when coexpressed with SOCS1. This effect was more pronounced in COS7 cells than in 293T cells and was dependent on the dose of cotransfected SOCS1 plasmid (Fig. 3*A* and data not shown). The capacity of SOCS1 to alter VAV protein expression was specific because other SOCS family proteins, SOCS2 or SOCS3, had no effect on the level of VAV expression (Fig. 3*B*). To assess the effects of SOCS1 on VAV protein turnover, COS7 cells were tranfected with VAV in the presence or absence of SOCS1. Metabolically ^{35}S-labeled VAV and onco-VAV immunoprecipitated after various chase times were separated by SDS-PAGE

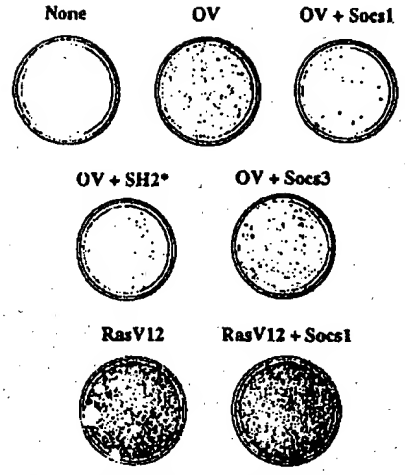

FIG. 2. **SOCS1 reduces onco-VAV-induced foci formation.** Untransfected NIH 3T3 cells (*None*) or NIH 3T3 cells tranfected with plasmids encoding the indicated proteins were grown to confluence and stained with Giemsa stain to visualize the foci. Plates from a representative experiment are shown. *OV*, onco-VAV; *SH2**, SOCS1 with R105K substitution in the phosphotyrosine binding pocket of the SH2 domain.

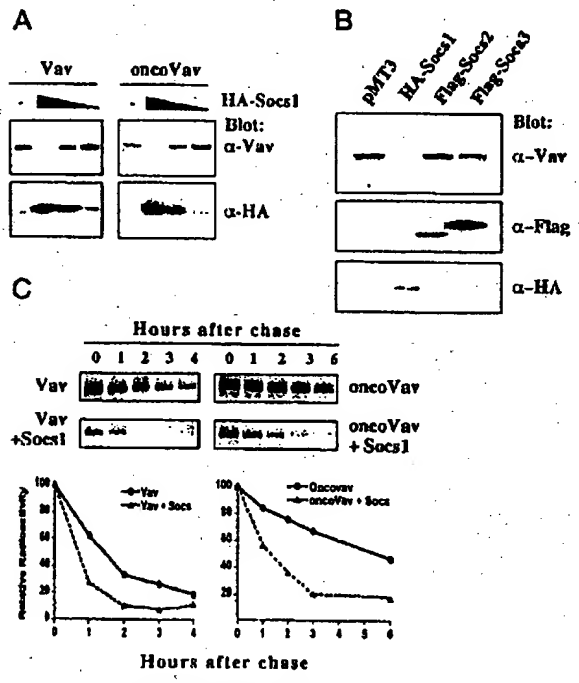

FIG. 3. *A*, SOCS1 destabilizes VAV and onco-VAV proteins. A constant amount of VAV or onco-VAV expression plasmid (0.5 μg) was transfected into COS7 cells with increasing amounts of SOCS1 expression plasmid (0, 0.11, 0.33, or 1 μg). VAV, onco-VAV, and SOCS1 expression were evaluated by Western blotting. *B*, down-regulation of VAV expression by SOCS1 is specific. COS7 cells were transfected with VAV in combination with HA-tagged SOCS1 or Flag-tagged SOCS2 or SOCS3. Total cell lysates were probed for VAV, SOCS1, SOCS2, and SOCS3 proteins by Western blotting. *C*, SOCS1 decreases the half-lives of VAV and onco-VAV proteins. COS7 cells were cotransfected with VAV or onco-VAV and SOCS1 or the empty vector. After 36 h, the cells were labeled with ^{35}S-Cys and -Met for 20 min and chased for the indicated times. VAV and onco-VAV were immunoprecipitated, separated in 10% SDS-PAGE, and autoradiographed. The incorporated radioactivity remaining after different chase period was measured by PhosphorImager to estimate protein stability.

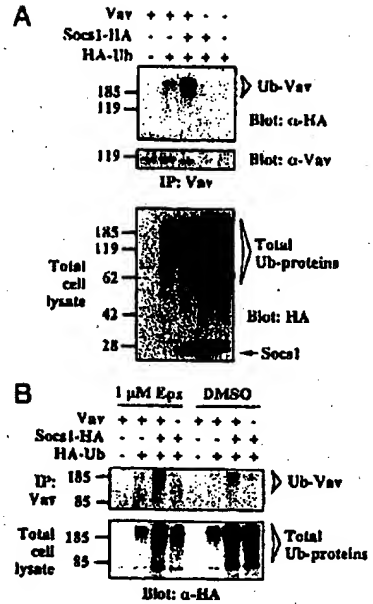

FIG. 4. *A*, SOCS1 potentiates VAV ubiquitination. Indicated combinations of VAV, HA-SOCS1, and HA-ubiquitin (*HA-Ub*) were co-expressed in COS7 cells. Cell lysates were denatured, VAV was immunoprecipitated (*IP*), and ubiquitinated VAV was detected by Western blotting with anti-HA antibody (*top panel*). Ubiquitinated VAV migrates as a high molecular weight smear because of the covalent addition of multiple ubiquitin moieties. The membrane was stripped and reprobed for VAV using anti-VAV antibody (*middle panel*). Ubiquitination of total cellular proteins is shown in the *bottom panel* by probing the whole cell lysate with anti-HA; expression of SOCS1 is indicated. *B*, proteasomal inhibitor stabilizes ubiquitinated VAV. Proteasomal inhibitor epoxomycin (*Epx*, 1 μM) or carrier dimethyl sulfoxide (*DMSO*) was added to the transfected cultures 8 h before detergent lysis. Other procedures are the same as described in *A*.

and visualized by fluorography (Fig. 3*C*). We observed that onco-VAV had an extended half-life (5.2 h) compared with VAV (1.4 h) and that in both cases SOCS1 increased the rate of degradation of VAV proteins. In the presence of SOCS1, the measured half-life of onco-VAV was 1.2 h and of VAV was 0.5 h.

Ubiquitination of VAV Is Increased by SOCS1—A number of signaling proteins have been shown to be down-regulated through proteolytic degradation pathways involving the ubiquitin and the 26S proteasome machinery (20). To test for whether SOCS1 might influence ubiquitination of VAV, we expressed HA-tagged ubiquitin together with VAV and SOCS1 in COS7 cells. Western blotting the immunoprecipitated VAV with anti-HA antibody showed that co-expression of SOCS1 results in a marked increase of VAV ubiquitination (Fig. 4*A*, *top panel*). Probing whole cell lysates with the anti-HA antibody revealed comparable levels of total ubiquitinated proteins in cells expressing SOCS1 and ubiquitin (Fig. 4*A*, *bottom panel*). Similar results were obtained for onco-VAV (data not shown). Further, the addition of the proteasomal inhibitor epoxomycin resulted in the accumulation of ubiquitinated VAV (Fig. 4*B*). These results indicate that SOCS1 couples VAV to the ubiquitination machinery and targets the ubiquitinated VAV to proteasomal degradation.

DISCUSSION

In this report we ascribe a new mechanism whereby the SOCS1 protein negatively regulates the VAV guanine nucleotide exchange factor by facilitating its degradation. This inhibitory function is distinct from its role as an inhibitor of the JAK family of tyrosine kinases. Insight into the mechanism by

which SOCS1 targets VAV for ubiquitination and destruction comes from the recent finding that SOCS1 and SOCS3 interact with the elongin BC complex (21, 22). Elongin BC also interacts with the tumor suppressor protein VHL to form an E3 ubiquitin ligase complex called VCB (VHL/elonginC/B) (23, 24). VHL binds to elongin C through an intermolecular four-helical bundle structure formed from one helix of elongin C and the three helices donated by VHL (25). The VHL α-helices that bind to elongin C are present as homologous structures contained within the COOH-terminal SOCS box of SOCS1 and all other SOCS family proteins (4, 21, 25). VHL functions as an adapter molecule recruiting specific proteins fated for ubiquitin-mediated degradation such as the hypoxia-inducible factor, HIF-1α, to the VCB E3 ligase (26). We propose that different SOCS box proteins have a function similar to VHL and recruit distinct sets of protein substrates such as VAV to the VCB-like E3 ligase complexes, where the substrates are ubiquitinated and targeted to proteasomal degradation. In support of this notion, another SOCS family protein, CIS, appears to suppress erythropoietin signaling by binding to the activated erythropoietin receptor and targeting it for ubiquitin-dependent degradation (27). It remains to be seen whether other SOCS1-binding proteins such as JAK, KIT, GRB2, p85, or ITK are similarly ubiquitinated and destabilized by SOCS1.

Two other proteins that have been reported to negatively regulate VAV function are Cbl-b and hSIAH2 (28, 29). VAV can bind to both c-Cbl and Cbl-b through its COOH-terminal SH3-SH2-SH3 region (28). Transfection experiments have shown that Cbl-b inhibits onco-VAV-mediated activation of JNK (28). c-Cbl has recently been defined as an E3 ligase that promotes ligand-induced ubiquitination of the epidermal growth factor receptor (30). The role of the Cbl family in regulating VAV protein turnover has not yet been examined. hSIAH2, a RING-finger containing human homolog of *Drosophila* Seven in absentia (SINA), binds to the COOH-terminal SH3-SH2-SH3 region of VAV and inhibits onco-VAV-induced activation of JNK (29). hSIAH2 appears to be a component of an E3 ubiquitin ligase protein complex that targets the tumor suppressor protein DCC and the N-CoR protein (31, 32). Unlike SOCS1, however, coexpression of hSIAH2 with VAV does not destabilize VAV (29).

SOCS1 suppresses cytokine signaling by inhibiting JAK. In this report, we have provided evidence for a novel function of SOCS1 to recruit signaling molecules such as VAV via dedicated protein-protein interaction domains for ubiquitin-mediated protein destruction. Validation of this model will require *in vitro* reconstitution of the ubiquitin ligase activity of the VAV-SOCS-VCB-like complex using recombinant components.

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Negative regulation of FAK signaling by SOCS proteins

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Focal adhesion kinase (FAK) becomes activated upon integrin-mediated cell adhesion and controls cellular responses to the engagement of integrins, including cell migration and survival. We show here that a coordinated signaling by integrins and growth factor receptors induces expression of suppressor of cytokine signaling-3 (SOCS-3) and subsequent interaction between endogenous FAK and SOCS-3 proteins in 3T3 fibroblasts. Cotransfection studies demonstrated that SOCS-3, and also SOCS-1, interact with FAK in a FAK-Y397-dependent manner, and that both the Src homology 2 (SH2) and the kinase inhibitory region (KIR) domains of the SOCS proteins contribute to FAK binding. SOCS-1 and SOCS-3 were found to inhibit FAK-associated kinase activity *in vitro* and tyrosine phosphorylation of FAK in cells. The SOCS proteins also promoted polyubiquitination and degradation of FAK in a SOCS box-dependent manner and inhibited FAK-dependent signaling events, such as cell motility on fibronectin. These studies suggest a negative role of SOCS proteins in FAK signaling, and for a previously unidentified regulatory mechanism for FAK function.

Keywords: adhesion/integrin/kinase/migration/signaling

Introduction

Focal adhesion kinase (FAK) is a ubiquitously expressed non-receptor protein tyrosine kinase that has emerged as a crucial molecule in integrating signals from integrins and receptor tyrosine kinases in processes such as cell survival, proliferation and motility (Schlaepfer *et al.*, 1999; Schaller, 2001). Genetic ablation of FAK results in early embryonic lethality in mice, and cells derived from knockout embryos demonstrate severe migration and survival defects (Ilic *et al.*, 1995; Ilic *et al.*, 1998; Sieg *et al.*, 1998; Owen *et al.*, 1999). Conversely, enhanced FAK signaling increases cell motility and promotes cell survival in an anchorage-independent manner (Cary *et al.*, 1996; Frisch *et al.*, 1996). Along these lines, a number of groups have reported that FAK protein levels and/or its activity are upregulated in invasive cancer cells (Jones *et al.*, 2000).

Ligand binding of integrins results in catalytic activation of FAK and in its autophosphorylation at Y397, which serves as a binding site for several Src homology 2 (SH2) domain-containing proteins, including Src kinases. The FAK–Src dual kinase complex leads to further phosphorylation of FAK, and also to phosphorylation and activation of a number of cytoskeleton-linked proteins, which transduce integrin-generated signals to downstream pathways, such as the mitogen-activated protein (MAP) kinase cascades, and to control of cell survival, motility and proliferation (Hanks and Polte, 1997; Schlaepfer *et al.*, 1999; Schaller, 2001). At present, the molecular mechanisms that control FAK activation remain largely unknown.

Suppressors of cytokine signaling (SOCS) are a family of proteins that act in a feedback loop to inhibit cytokine responses and activation of the JAK/STAT pathway in hematopoietic cells (Naka *et al.*, 1999; Yasukawa *et al.*, 2000; Krebs and Hilton, 2001). Evidence indicates that SOCS proteins modulate other signaling pathways, as well. Cacalano *et al.* (2001) reported recently that SOCS-3 participates in regulation of signaling by various growth factors in non-hematopoietic cells. The SOCS-proteins are structurally characterized by distinct domains that include a central SH2 domain and a carboxyl-terminal homology region termed SOCS box. The SH2 domain of SOCS-proteins interacts with phosphorylated tyrosine residues in tyrosine kinases and, in many cases, negatively regulates their activity (Yasukawa *et al.*, 2000; Krebs and Hilton, 2001). The SOCS boxes of SOCS-1 and SOCS-3 have been found to mediate interaction with the elongin B/C complex (Kamura *et al.*, 1998; Zhang *et al.*, 1999). The elongin B/C complex was originally identified as a component of the von Hippel-Lindau tumor suppressor E3 ligase complex, which also contains RING finger protein Rbx1 and Cullin-2 (Ivan and Kaelin, 2001). Thus, the SOCS box may act as a bridge between SOCS-SH2 interacting proteins and E3 ubiquitin ligases, and regulate protein turnover by targeting proteins for polyubiquitination and proteasome-mediated degradation. For SOCS-1, there is direct evidence that it can ubiquitinate and regulate the half life of Vav (De Sepulveda *et al.*, 2000) and the JAK tyrosine kinases (Frantsve *et al.*, 2001; Kamizono *et al.*, 2001; Ungureanu *et al.*, 2002).

We report here that a coordinated signaling by integrins and growth factor receptors results in upregulation of SOCS protein expression and subsequent interaction of SOCS proteins with FAK in 3T3 fibroblasts. Our studies further demonstrate that SOCS-1 and SOCS-3 bind to tyrosine-phosphorylated FAK in a FAK-Y397-dependent manner, and inhibit FAK-associated kinase activity *in vitro* and tyrosine phosphorylation of FAK in cells. SOCS-1 and SOCS-3 were also found to facilitate ubiquitination and degradation of FAK in a SOCS box-dependent manner and to inhibit FAK-induced MAPK activation and cell motility. These studies identify SOCS proteins as potential novel regulators of FAK signaling.

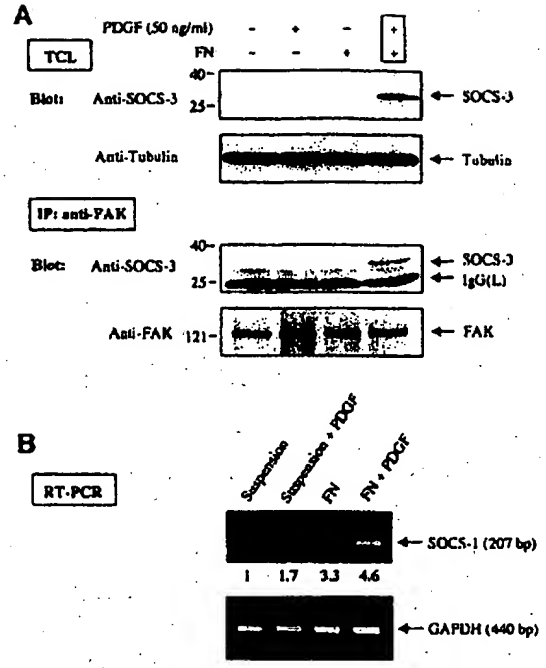

Fig. 1. (A) A coordinate signaling by integrins and PDGF receptor results in SOCS expression and SOCS–FAK interaction. Serum-starved NIH 3T3 cells were detached, stimulated or not with 50 ng/ml PDGF and either kept in suspension or replated on FN-coated dishes for 1 h. Cell lysates were subjected to anti-SOCS-3 immunoblot analysis, and to anti-tubulin immunoblotting to confirm equal loading (top two panels). Cell lysates prepared as above were also subjected to immunoprecipitation analysis with anti-FAK antibody followed by immunoblot analysis with anti-SOCS-3 antibody. The same membrane was stripped and reprobed with anti-FAK antibody to confirm equal loading (bottom two panels). TCL, total cell lysate. (B) Integrin and PDGF receptor signaling enhances SOCS-1 mRNA levels. Cells were treated as above, and total RNA was isolated. The levels of SOCS-1 mRNA, and GAPDH levels as a control, were analyzed by a semi-quantitative RT–PCR analysis as described in Materials and methods.

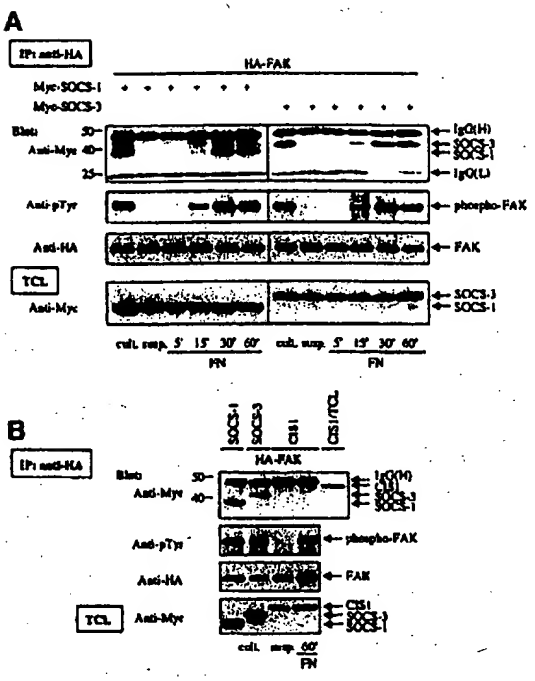

Fig. 2. Adhesion-dependent interaction of SOCS-1 and SOCS-3 with FAK. (A) COS-7 cells were transiently transfected with the indicated plasmids (0.2 μg of HA-FAK, 0.5 μg of Myc-SOCS-1/3). Twenty-four hours after transfection, cells were serum-starved for additional 24 h, detached, kept in suspension for 30 min ('susp.'), and replated or not on FN for the indicated times. Alternatively, cells were lysed as a monolayer culture 48 h after transfection without prior additional manipulation ('cult.'). Cell lysates were immunoprecipitated with anti-HA antibody, and the precipitates were analyzed by immunoblotting with antibodies against Myc, phosphotyrosine (pTyr) or HA. Total cell lysates were subjected to immunoblotting with anti-Myc antibodies to confirm SOCS-protein expression levels. (B) COS-7 cells were transiently transfected with the indicated plasmids (0.2 μg of HA-FAK, 0.5 μg of Myc-SOCS-1/3 or CIS1). Twenty-four hours after transfection, CIS1-FAK-cotransfected cells were serum-starved for additional 24 h, detached, kept in suspension for 30 min, and replated or not on FN for 1 h. FAK-SOCS-1/3-cotransfected cells were treated as described above for monolayer culture. Cell lysates were immunoprecipitated with anti-HA antibody, and the precipitates were analyzed by immunoblotting with antibodies against Myc, pTyr or HA. An aliquot of total cell lysate from CIS1-transfected cells was included as a control in the anti-Myc immunoblot of anti-HA immunoprecipitates to determine the motility of the CIS1 protein relative to the IgG band. Total cell lysates of all samples were subjected to immunoblot with anti-Myc antibody to confirm SOCS-protein expression levels.

Results

Coordinate signaling by integrins and growth factor receptors results in SOCS expression and SOCS–FAK interaction

It was reported recently that treatment of adherent cells, including NIH 3T3 fibroblasts, with growth factors such as PDGF and EGF results in a rapid induction of SOCS-3 (Cacalano *et al.*, 2001). We also noticed a report in which Pyk2, a close homolog of FAK, had been tested in a yeast-two hybrid experiment and found to interact with SOCS-1 and SOCS-3, but not with several other members of the SOCS family (Masuhara *et al.*, 1997). These observations prompted us to examine the intriguing possibility that SOCS proteins might have a previously unidentified role in regulating FAK signaling. To this end, serum-starved monolayer cultures of NIH 3T3 cells were detached, stimulated or not with PDGF, and either kept in suspension or replated for 1 h on dishes that had been coated with the integrin ligand fibronectin (FN). As shown in Figure 1A (upper panels), simultaneous activation of integrin and growth factor pathways resulted in a rapid induction of SOCS-3 protein levels. While unavailability of suitable antibodies prohibited us from examining the protein levels of SOCS-1, a robust, 5-fold induction in the SOCS-1 mRNA levels was observed in 3T3 cells that had been plated on FN and treated with PDGF, compared with cells that had been kept in suspension (Figure 1B). In order to examine the potential interaction between endogenous FAK and SOCS-3 proteins, anti-FAK immunoprecipitation was carried out followed by immunoblotting with anti-SOCS-3 antibodies. A coordinate signaling by integrins and PDGF resulted in an interaction between the endogenous FAK and SOCS-3 proteins in NIH 3T3 cells (Figure 1A).

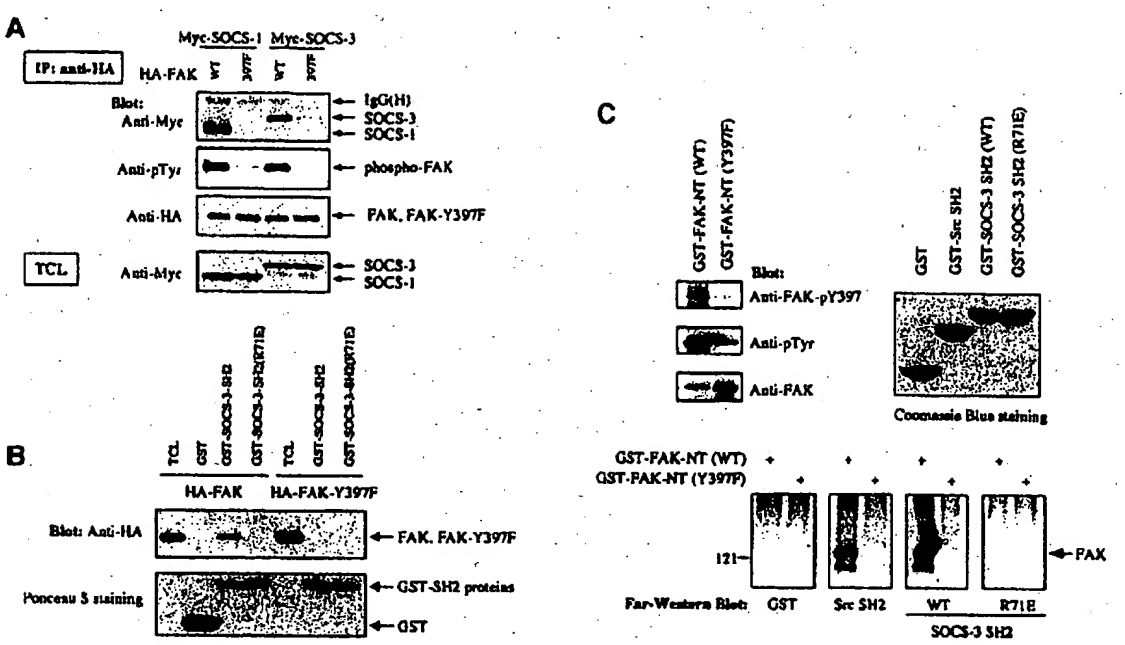

Fig. 3. Y397 in FAK regulates the FAK–SOCS interaction. (A) COS-7 cells were transiently transfected with the indicated plasmids (0.2 μg of HA-FAK or HA-FAK-Y397F, 0.5 μg of Myc-SOCS-1/3). Forty-eight hours after transfection, cell lysates were immunoprecipitated with anti-HA antibody, and the precipitates were analyzed by immunoblotting with antibodies against Myc, pTyr or HA. Total cell lysates were subjected to immunoblotting with anti-Myc antibody to confirm SOCS protein expression levels. (B) Denatured lysates of HA-FAK or HA-FAK-Y397F-transfected COS-7 cells were incubated with purified GST or with GST–SOCS-3-SH2 fusion proteins conjugated to glutathione–Sepharose beads. Associated proteins were examined by anti-HA immunoblotting (top panel). Ponceau S staining of the same blot is shown on the bottom panel. (C) GST fusion proteins coding for the N-terminal domain (amino acids 1–406) of wild-type or Y397-mutant of FAK were produced in the bacterial TK strain expressing an active tyrosine kinase. The produced proteins were separated by SDS–PAGE and transferred onto PVDF membrane and analyzed by immunoblotting with the indicated antibodies (upper left panel), or by far-western blotting with the indicated fusion proteins as described in Materials and methods (lower panel). Coomassie Blue staining of the fusion proteins used as probes is shown on the upper right panel.

In order to study the mechanism of SOCS-3 and FAK interaction in more detail, Myc-tagged SOCS-3 was transiently expressed in COS-7 cells, and its interaction with FAK in suspended and FN-plated cells was examined. We also examined the potential interaction between transfected, Myc-tagged SOCS-1 and FAK. As shown in Figure 2A, transfected SOCS-1 and SOCS-3 readily interacted with FAK in cells that had been plated on FN, but not in cells that had been kept in suspension. Another SOCS protein, CIS1, failed to interact with FAK in both suspended and adherent cells (Figure 2B). Thus, our results demonstrate that integrin-mediated signaling contributes to the induction of SOCS family members upon growth factor stimulation, and also regulates the interaction between FAK and exogenously expressed SOCS-1 and SOCS-3 proteins.

Y397 in FAK and the KIR- and SH2-domains of SOCS proteins participate in the FAK–SOCS interaction

Previous studies have indicated that the SH2 domain and an adjacent N-terminal region named the KIR domain mediate the binding of SOCS-proteins to activated and tyrosine-phosphorylated JAK kinases (Nicholson *et al.*, 1999; Sasaki *et al.*, 1999; Yasukawa *et al.*, 1999). Ligation of integrins with matrix proteins results in a rapid activation and tyrosine phosphorylation of FAK, which, as shown in Figure 2A, correlated with the binding of FAK to SOCS-1 and SOCS-3. This finding suggested that similar to JAKs, FAK may interact with SOCS proteins in an activation- and tyrosine phosphorylation-dependent manner. To investigate this further, a form of FAK in which the main autophosphorylation site, Y397, had been mutated to phenylalanine (FAK-Y397F) was transfected into COS cells and its interaction with cotransfected SOCS-1 and SOCS-3 was studied. As shown in Figure 3A, FAK-Y397F demonstrated little tyrosine phosphorylation in adherent COS cells, and in contrast to wild-type FAK, it failed to interact with cotransfected SOCS-1 and SOCS-3 proteins.

That SOCS proteins would interact with a tyrosine-phosphorylated form of FAK was further supported by two additional lines of investigation. First, we found that a GST fusion protein of the SH2 domain of SOCS-3 readily precipitated the wild-type, but not the Y397F-mutant form of FAK in denatured cell lysates, while a SOCS-3 fusion protein with an inactive SH2-domain (R71E) failed to do so (Figure 3B). Second, we carried out a far-western blot analysis to further demonstrate that the FAK–SOCS interaction is dependent on the FAK-Y397 and the SH2 domain of the SOCS protein, and to explore the possibility that the two proteins might interact directly. As shown in Figure 3C, this analysis indicated that the wild-type form of SOCS-3-SH2, but not the R71E-mutant form was

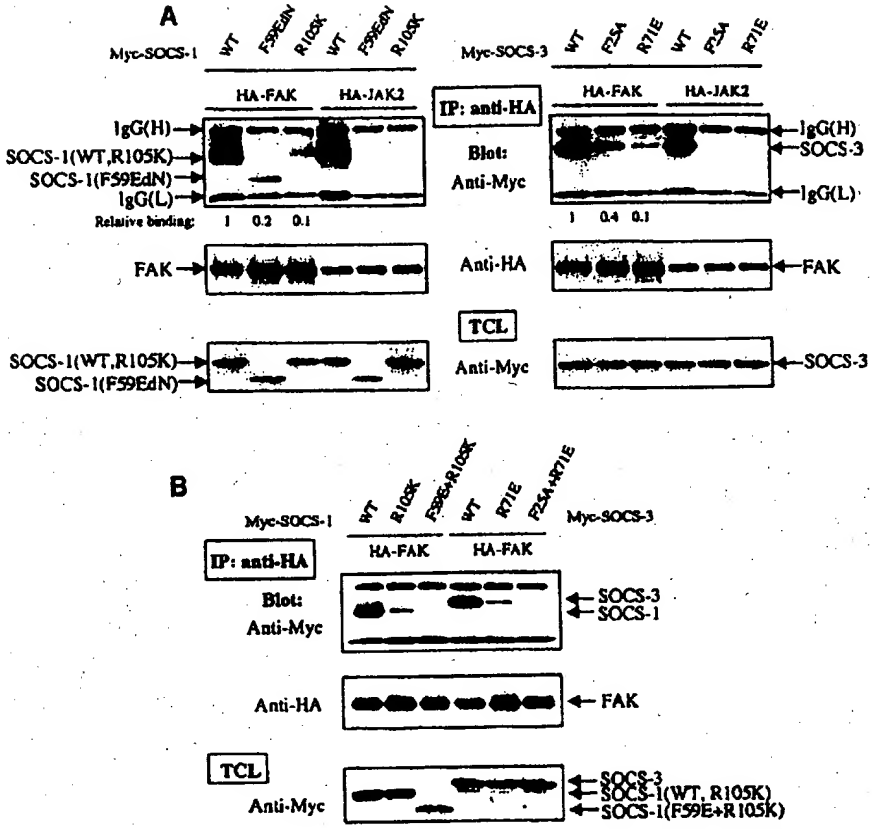

Fig. 4. The KIR- and SH2-domains of SOCS proteins participate in SOCS–FAK interaction. (A and B) COS-7 cells were transiently transfected with indicated plasmids (0.2 μg of HA-FAK or HA-JAK2, 0.5 μg of Myc-SOCS-1/3). Forty-eight hours after transfection, cell lysates were immunoprecipitated with anti-HA antibody, and the precipitates were analyzed by immunoblotting with antibodies against Myc or HA. Total cell lysates were subjected to immunoblot with anti-Myc antibody to confirm SOCS protein expression levels. The relative FAK-SOCS interaction was determined by a densitometric analysis and the NIH Image 1.62 program. The KIR-mutant form of SOCS-1 (SOCS-1-F59EdN) and the SOCS-1 double-mutant (SOCS-1-F59E, R105K) contain a deletion of the first 51 amino acids that has been shown to stabilize the expressed protein without changing its binding affinity or specificity (Yasukawa *et al.*, 1999).

capable of binding to bacterially produced and tyrosine-phosphorylated FAK N-terminal fusion protein (FAK-NT, amino acids 1–406). The SH2 domain of SOCS-3 failed to associate with the Y397F mutant form of FAK-NT, suggesting that at least *in vitro*, the SH2-domain of SOCS-3 directly interacts with FAK in Y397-dependent manner.

We next expressed forms of SOCS-1 or SOCS-3 in which either the KIR domain (F59E and F25A mutants, respectively) or the SH2-domain (R105K and R71E mutants, respectively) had been inactivated. Similar to what has been reported previously, we found that intact KIR- and SH2-domains are necessary for JAK2 to interact with SOCS-1 and SOCS-3 (Figure 4A). When coexpressed with FAK, the mutant SOCS-proteins demonstrated a greatly reduced, but detectable interaction with FAK. Importantly, however, double KIR/SH2-mutant forms of SOCS-1 and SOCS-3 failed to interact with FAK (Figure 4B), suggesting that the KIR- and SH2-domains of SOCS-proteins mediate binding to FAK. Whether these results are reflective of a quantitative and/or qualitative difference in the mechanism by which SOCS proteins interact with JAK and FAK kinases remains to be determined.

SOCS proteins inhibit FAK-associated kinase activity in vitro, and tyrosine phosphorylation of FAK in cells

SOCS-1 and SOCS-3 are known to inhibit the catalytic activity of JAK2 by binding through the KIR and SH2-domains (Nicholson *et al.*, 1999; Sasaki *et al.*, 1999; Yasukawa *et al.*, 1999);we therefore examined the effect of SOCS proteins on the catalytic activity and phosphorylation of FAK *in vitro* and in cells. FAK was coexpressed or not with SOCS-1 and SOCS-3 and FAK immunoprecipitates were subjected to an *in vitro* kinase assay. As shown in Figure 5A, the capability of immunoprecipitated FAK to catalyze its own phosphorylation or phosphorylation of the exogenous substrate poly(Glu:Tyr) was greatly reduced upon coexpression of SOCS-1 or SOCS-3. The reduced *in vitro* catalytic activity correlated with reduced tyrosine phosphorylation of FAK in cells. When increas-

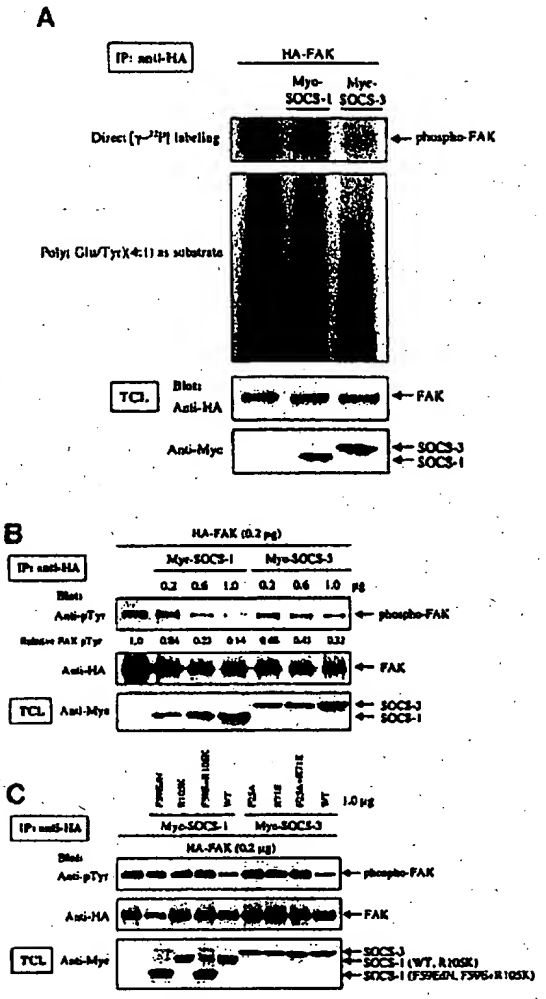

Fig. 5. SOCS-proteins inhibit FAK-associated kinase activity *in vitro* and tyrosine phosphorylation of FAK in cells. (A) COS-7 cells were transfected with HA-FAK (0.5 μg) with or without Myc-SOCS-1 or SOCS-3 (1.0 μg). Twenty-four hours after transfection, cells were serum-starved for an additional 24 h, lysed and the lysates were subjected to anti-HA immunoprecipitation. An *in vitro* kinase assay was carried out as described in Materials and methods. Immunoblotting of the anti-HA immunoprecipitates with anti-HA antibody, and immunoblotting of total cell lysates with anti-Myc antibody are shown in the bottom panels. (B and C) COS-7 cells were transiently transfected with 0.2 μg of HA-FAK with or without increasing concentrations of wild-type Myc-SOCS-1 or Myc-SOCS-3 (0, 0.2, 0.6 and 1.0 μg) (B), or 1.0 μg of the indicated SOCS mutants (C). Twenty-four hours after transfection, cells were serum-starved for an additional 24 h, lysed and the lysates were subjected to anti-HA immunoprecipitation. Equal amounts of cell lysates were immunoprecipitated with anti-HA antibody, followed by immunoblotting with anti-pTyr and anti-HA antibodies. The relative FAK phosphorylation was determined in (B) as above. Total cell lysates were subjected to immunoblot with anti-Myc antibody to confirm SOCS protein expression levels.

ing amounts of SOCS-1 and SOCS-3 proteins were expressed in COS cells, a dose-dependent decrease in the tyrosine phosphorylation of FAK was observed (Figure 5B). Importantly, the KIR- and SH2-mutant forms of SOCS-1 and SOCS-3 failed to have a significant effect on FAK tyrosine phosphorylation (Figure 5C). Thus, these studies suggest that SOCS-1 and SOCS-3 regulate FAK activity and phosphorylation both *in vitro* and in cells, and that this regulation correlates with the capability of the SOCS proteins to interact with FAK.

SOCS proteins induce proteasome-dependent degradation and SOCS box-dependent ubiquitination of FAK

As noted above, SOCS proteins have been reported to induce proteasome-dependent degradation of their target proteins. During the course of these studies, we observed that when wild-type SOCS-1 or SOCS-3 proteins were overexpressed with FAK, the amounts of FAK protein in cell lysates were reduced (see e.g. Figure 5B). Metabolic labeling and pulse–chase experiments were therefore conducted to investigate the effect of SOCS-1 and SOCS-3 on the turnover of FAK. As shown in Figure 6, the turnover of the ^{35}S-labeled wild-type FAK protein was significantly enhanced upon coexpression of SOCS-1 and SOCS-3, while treatment of the cells with β-lactacystin, which is an irreversible proteasome inhibitor, stabilized the protein. The turnover of the FAK-Y397F mutant protein, which is incapable of interacting with SOCS-1 and SOCS-3, was not affected by the coexpression of the SOCS-proteins. These data suggest that SOCS proteins promote a proteasome-dependent degradation of FAK, and this effect correlates with the capability of the SOCS proteins to interact with FAK.

To investigate whether SOCS-induced FAK degradation was due to SOCS-mediated ubiquitination of FAK, we cotransfected FAK with wild-type and SOCS box-deleted forms of SOCS-1 and SOCS-3. To enhance tyrosine phosphorylation and subsequent interaction of FAK with the SOCS-proteins, the cells were pretreated with the phosphatase inhibitor pervanadate for 60 min; similar treatment was performed when ubiquitination of JAK2 by SOCS-1 was studied (Ungureanu *et al.*, 2002). In some experiments, treatment of cells with β-lactacystin was carried out to inhibit proteolytic degradation of ubiquitinated proteins. After immunoprecipitation, FAK immunocomplexes were dissociated followed by FAK reimmunoprecipitation to remove putative coimmunoprecipitated proteins from the complex. As shown in Figure 7A, little polyubiquitination of FAK was observed in the absence of coexpression of SOCS proteins. Importantly, coexpression of wild-type SOCS-1 and SOCS-3, but not their SOCS box-deleted forms, resulted in enhanced polyubiquitination of FAK, which was further augmented by pretreatment of the cells with β-lactacystin. SOCS box-dependent stimulation of FAK ubiquitination was also observed in *in vitro* ubiquitination assays, in which wild-type SOCS proteins but not their SOCS box-deletion mutants catalyzed polyubiquitination of either immunoprecipitated (Figure 7B) or *in vitro* transcribed and translated (Figure 7C) wild-type FAK protein, but not of the mutant FAK-Y397F protein.

We next examined whether the endogenous FAK protein is a target for SOCS-mediated polyubiquitination. Ubiquitin-transfected 3T3 cells were serum-starved and pretreated with pervanadate and MG132 to enhance FAK tyrosine phosphorylation and to inhibit proteolytic degradation of ubiquitinated proteins, respectively. The cells

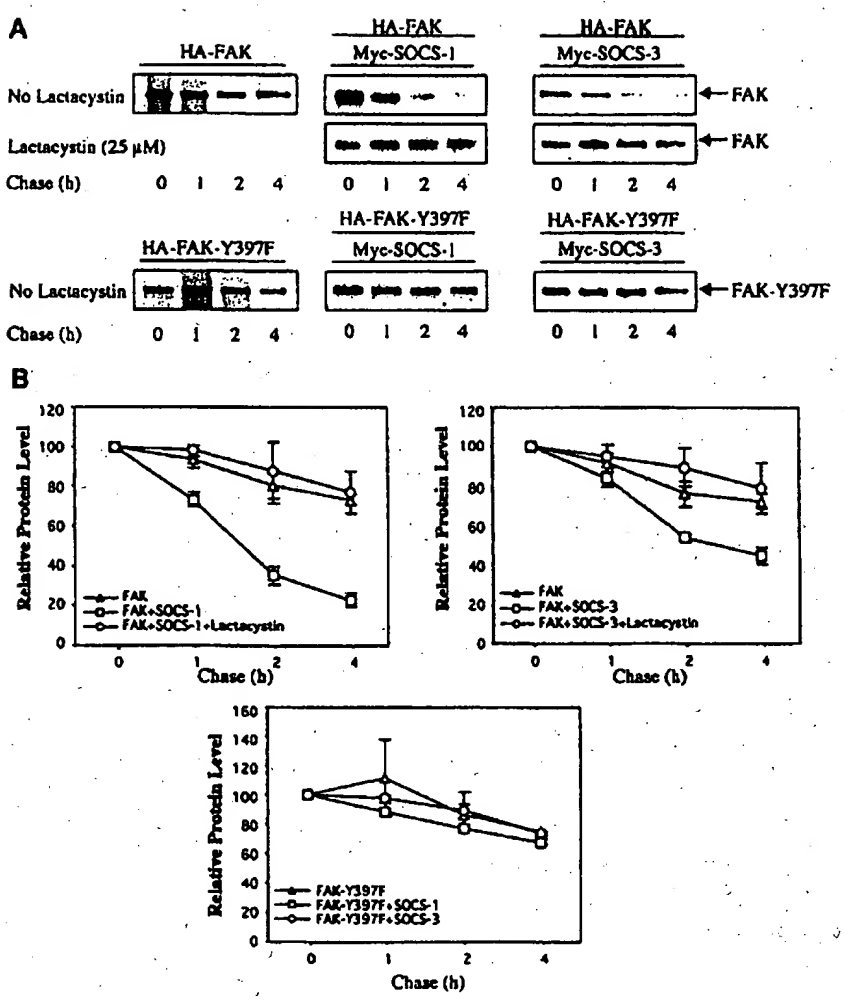

Fig. 6. SOCS-1 and SOCS-3 enhance the turnover of wild-type FAK but not of FAK-Y397F protein. (A) COS-7 cells were transfected with HA-FAK or HA-FAK-Y397F (0.5 μg) with or without Myc-SOCS-1 or Myc-SOCS-3 (1.0 μg). The transfected cells were pulse-labeled as described in Materials and methods followed by a chase for the indicated time periods. The ^{35}S-labeled FAK proteins were immunoprecipitated with anti-HA antibody, and subjected to SDS-PAGE followed by autoradiography. Where indicated, the proteasome inhibitor β-lactacystin at 25 μM was added to the cells 1 h before labeling and maintained in the medium throughout the pulse-chase experiment. A representative experiment is shown in (A). (B) Densitometric analysis of the blots of three individual experiments was carried out by using the NIH-Image 1.62 program. The relative protein amounts at time point zero were denoted as 100.

were then stimulated or not with PDGF, and either kept in suspension or replated on FN. As shown in Figure 7D, simultaneous stimulation of cells by integrin ligation and PDGF resulted in polyubiquitination of the endogenous FAK protein, and this coincided with induction of SOCS-3 protein expression and interaction between endogenous FAK and SOCS-3 proteins. Importantly, exogenously expressed SOCS-3ΔSB abolished polyubiquitination of FAK in FN-plated, PDGF-stimulated cells in a dominant-negative manner. Taken together, these results demonstrate that SOCS-1 and SOCS-3 induce polyubiquitination of FAK in cells in a SOCS box-dependent manner, and that this modification likely targets FAK for proteasome-mediated degradation.

SOCS proteins inhibit FAK-induced Erk activity and cell migration

As a corollary of the above findings, we next examined whether SOCS proteins regulate FAK-dependent cellular functions. It has been demonstrated previously that exogenous expression of FAK results in activation of the Erk MAP kinase cascade (Schlaepfer and Hunter, 1997), and we utilized this assay as a biochemical read out to examine the effect of SOCS proteins on FAK signaling. Similar to what was reported previously, we found that exogenous expression of FAK induced activation of Erk as measured by anti-phospho-MAPK immunoblotting (Figure 8A). Coexpression of SOCS-1 or SOCS-3 in turn significantly inhibited the capability of FAK to activate

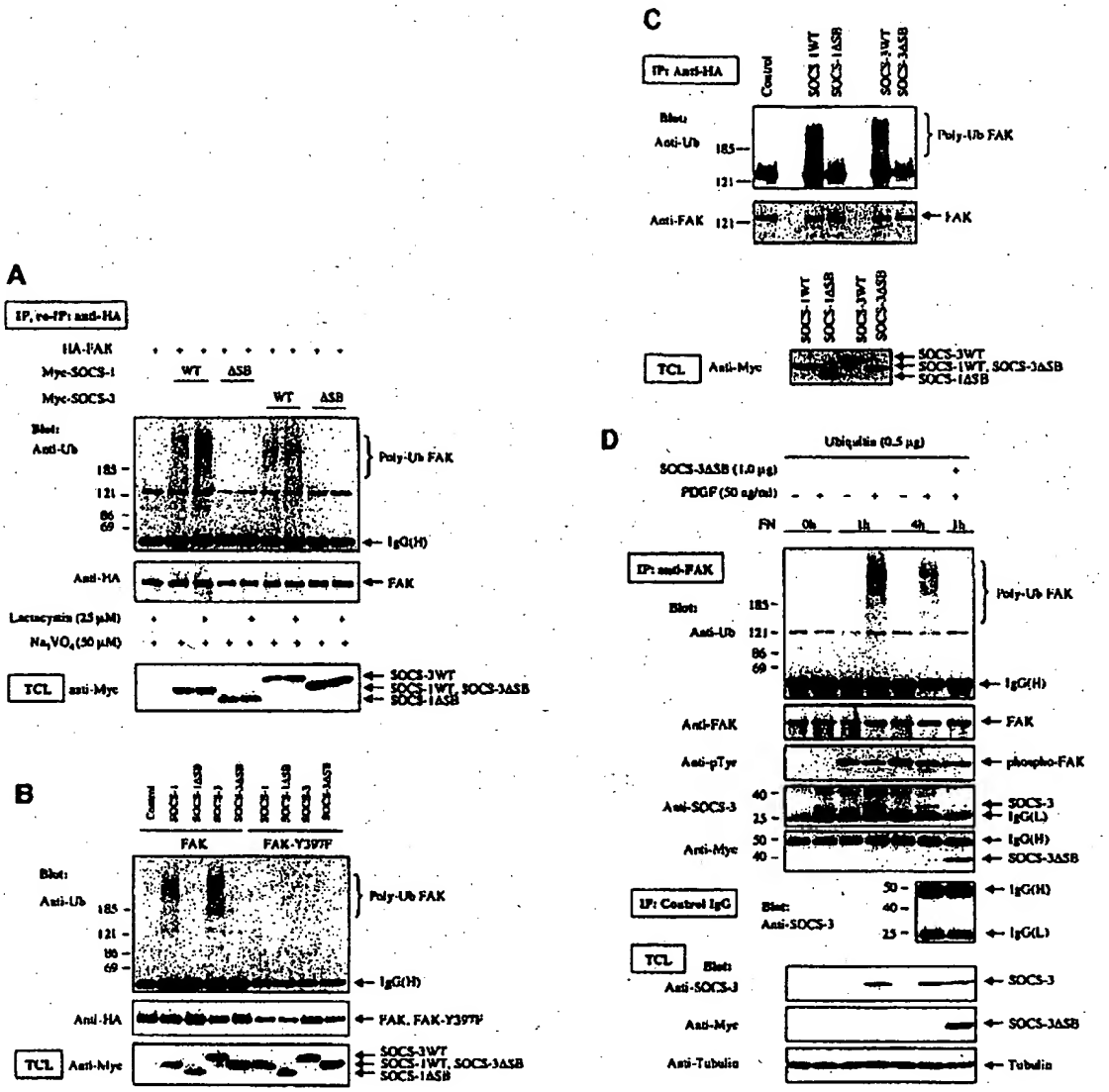

Fig. 7. SOCS-proteins promote polyubiquitination of FAK. (A) COS-7 cells were transiently transfected with HA-FAK (0.5 μg) alone or with wild-type forms of Myc-SOCS-1 or SOCS-3 or their SOCS box-deletion mutants (SOCSΔSB) (1.0 μg). Twenty-four hours after transfection the cells were serum-starved overnight and pretreated with or without β-lactacystin (25 μM) for 4 h prior to pervanadate treatment (50 μM) for 1 h. FAK was immunoprecipitated with an anti-HA antibody, followed by a dissociation and reprecipitation with the same antibody. FAK immunoprecipitates were analyzed by immunoblotting with antibodies against ubiquitin and HA. Total cell lysates were subjected to immunoblotting with anti-Myc antibody to confirm SOCS-protein expression levels. (B) *In vitro* ubiquitination reactions on immunoprecipitated wild-type FAK or FAK-Y397F mutant were performed as described in the Materials and methods in the presence or absence of cell lysates containing the indicated SOCS proteins. The reaction products were separated on SDS–PAGE, transferred onto PVDF membrane, and the blots were probed with anti-ubiquitin antibody (upper panel). Equal loading of the various SOCS proteins was verified by anti-Myc immunoblotting of total cell lysates (lower panel). (C) HA-tagged wild-type FAK was generated by a coupled *in vitro* transcription/translation system. An *in vitro* ubiquitination assay was performed on the Sepharose-purified FAK as in (B). (D) NIH 3T3 cells were transfected with 0.5 μg of plasmid coding for ubiquitin with or without 1.0 μg of SOCS-3ΔSB. Twenty-four hours after transfection, the cells were serum-starved overnight, detached, stimulated or not with PDGF, and either kept in suspension or replaced on FN for 1 or 4 h in the presence of 50 μM pervanadate and 25 μM proteasome inhibitor MG132. Cell lysates were subjected to immunoprecipitation with anti-FAK antibody followed by immunoblotting with antibodies against ubiquitin, FAK, pTyr, SOCS-3 and Myc (upper panels). An isotype-matched IgG was used as a control to demonstrate that anti-FAK antibodies do not unspecifically precipitate SOCS-3 (middle panel). Total cell lysates were subjected to immunoblot with anti-SOCS-3, anti-Myc and anti-tubulin antibodies (bottom three panels).

Erk. In a control experiment, SOCS-1 and SOCS-3 had no effect on the basal phosphorylation of Erk1 in COS cells.

As an additional functional read-out, we examined the capability of SOCS-proteins to modulate FAK-dependent cell motility. FAK was transiently expressed in NIH 3T3 cells and as demonstrated before (Cary *et al.*, 1996), its overexpression induced cell motility on FN. As shown in Figure 8B, coexpression of SOCS-1 and SOCS-3 signifi-

cantly inhibited FAK-stimulated cell motility. As an additional line of investigation, we studied the capability of SOCS-proteins to modulate PDGF-induced cell motility on FN. It has been shown previously that FAK integrates PDGF receptor and integrin signals to promote cell migration (Sieg *et al.*, 2000), and we found the same here by demonstrating that the dominant-negative molecule of FAK known as FRNK (Gilmore and Romer, 1996; Richardson and Parsons, 1996) inhibits PDGF-induced cell motility on FN (Figure 8C). Importantly, exogenous expression of SOCS-1 and SOCS-3 similarly resulted in a dose-dependent inhibition of PDGF-stimulated cell migration. Taken together, these studies demonstrate that exogenous expression of SOCS proteins results in an efficient inhibition of FAK-mediated biochemical and cell biological signaling events.

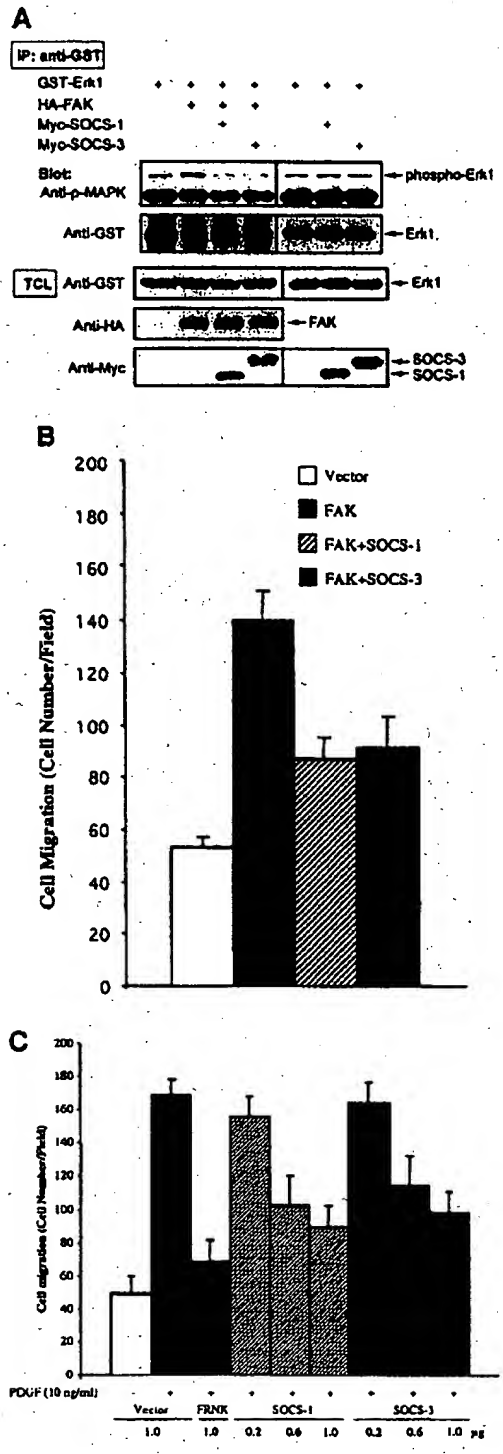

Fig. 8. SOCS proteins inhibit FAK-induced Erk activity and cell migration. (A) COS-7 cells were cotransfected with GST-Erk1 (1.0 μg) and HA-FAK (0.5 μg) with or without Myc-SOCS-1 or SOCS-3 (1.0 μg). Cell lysates were prepared 48 h after transfection, precipitated with anti-GST antibody, and the precipitates were analyzed by immunoblotting with anti-phospho-Erk antibody. The membrane was stripped and reprobed with anti-GST antibody to confirm equal loading. Total cell lysates were immunoblotted with antibodies against GST, HA and Myc (lower panels). Student's *t*-test demonstrated $P < 0.02$ between Erk activity in cells transfected with HA-FAK and HA-FAK + Myc-SOCS-1/3 in three independent experiments. (B) NIH 3T3 cells were transfected with an empty pcDNA3 vector, or with HA-FAK (0.5 μg) together with or without Myc-SOCS-1 or SOCS-3 (1.0 μg). A plasmid encoding β-galactosidase (0.2 μg) was included in all transfections. Haptotactic cell migration assay on FN was carried out as described in Materials and methods. (C) NIH 3T3 cells were transfected with pcDNA3 vector, HA-FRNK (1.0 μg), or increasing amounts of Myc-SOCS-1 or SOCS-3 (0.2, 0.6 or 1.0 μg). A vector coding for β-galactosidase (0.2 μg) was included in all transfections. Cells were added to the migration chambers and the number of cells that migrating towards PDGF (10 ng/ml) was determined 3 h later as described in Materials and methods.

Discussion

In contrast to the rapid progress that has been made in elucidating the FAK downstream signaling pathways, relatively little is known about the mechanisms of regulation of FAK activity. Our studies in this report demonstrate that the SOCS proteins may have a previously unidentified role in providing a negative regulatory mechanism for FAK function.

The expression of SOCS proteins is induced in response to cytokine stimulation, and overexpression of these proteins results in inhibition of cytokine signaling (Naka *et al.*, 1999; Yasukawa *et al.*, 2000; Krebs and Hilton, 2001). Our studies demonstrate an essential role for integrin-mediated cell adhesion in contributing to the rapid upregulation of SOCS-3 protein levels by PDGF in 3T3 fibroblasts. Thus, these results identify a novel mechanism for cross-talk between integrins and receptor tyrosine kinases; it remains to be determined whether integrin ligation regulates SOCS protein expression in response to growth factor and cytokine stimulation in other cell types. The pathways downstream of integrins and PDGF receptor that lead to the induction of SOCS-3 are presently unknown.

Our studies demonstrate that integrin-mediated cell adhesion regulates the interaction between FAK and the SOCS proteins. We found that the interaction is dependent on the major autophosphorylation site, Y397, in FAK, suggesting that SOCS-1 and SOCS-3 specifically interact with and target activated FAK. We further found that the

SH2-domain of SOCS-3 was able to interact with FAK in denatured cell lysates and in a far-western experiment. These results are suggestive of a direct interaction between FAK and SOCS proteins. Our studies further demonstrated that the KIR domain within the SOCS proteins also binds to FAK (or FAK protein complex), but the exact mechanism of this interaction remains to be determined.

It has been shown that autophosphorylation of FAK on Y397, subsequent binding of Src kinase to this site, and further phosphorylation of FAK in the activation loop by Src are required for full FAK activation (Calalb *et al.*, 1995). Conceivably, binding of SOCS proteins to Y397 could prevent full activation of FAK by Src, which could result in the observed decrease in FAK kinase activity and phosphorylation upon SOCS protein expression. Alternatively (or at the same time), SOCS protein-mediated displacement of Src from FAK in and of itself could account for the decreased activity observed in the kinase assay, decreased phosphorylation of FAK in cells, and decreased activation of FAK-dependent biochemical and biological signaling events. Previous studies have demonstrated that FAK acts as a docking protein to recruit Src to phosphorylate intracellular substrate proteins (Ruest *et al.*, 2001), and in many cell systems, it is the FAK–Src dual-kinase complex, rather than FAK activity *per se*, that is functionally significant (Schlaepfer *et al.*, 1999).

Our studies do not preclude the possibility that SOCS-proteins could interact with other sites in FAK, which become phosphorylated in a Y397-dependent manner *in vivo*. Along these lines, tyrosine phosphorylation of FAK at Y397 is required for Src-mediated phosphorylation of Y576/577 in the activation loop of FAK (Calalb *et al.*, 1995). Interestingly, SOCS-1 and SOCS-3 have been shown to bind to a phosphorylated Y1007 in the activation loop of JAK2 and inhibit its kinase activity (Nicholson *et al.*, 1999; Sasaki *et al.*, 1999; Yasukawa *et al.*, 1999); a similar mechanism of binding and inhibition could apply to FAK. Additionally, Y397-dependent phosphorylation of FAK is known to result in binding of FAK to various SH2-domain containing proteins (Schlaepfer *et al.*, 1999; Schaller, 2001). Some of these proteins, such as Grb2, have been shown to interact with SOCS-proteins (De Sepulveda *et al.*, 1999) and could therefore function as a bridge between FAK and SOCS proteins.

Our results indicate that SOCS proteins not only regulate tyrosine phosphorylation of FAK, but they also promote its proteasome-mediated degradation. To our knowledge, these studies are the first to demonstrate that FAK is a target for polyubiquitination-induced degradation. Our functional studies indicated that exogenous expression of SOCS proteins results in an efficient downregulation of FAK-mediated cellular functions, such as cell motility; whether this downregulation requires both the kinase inhibitory and the polyubiquitination activity of SOCS proteins remains to be determined. At present, little is known about the molecular mechanisms that regulate FAK function *in vivo*. A novel protein inhibitor FIP200 has been recently shown to bind to the kinase domain of FAK and to inhibit its kinase activity upon coexpression (Abbi *et al.*, 2002). Numerous phosphatases have been suggested as regulators of FAK, and FAK has also been shown to be a target for proteolytic cleavage by caspases and calpain (Schlaepfer *et al.*, 1999; Schaller, 2001). Whether cleavage of FAK by these proteases simply terminates signaling or generates fragments that transmit aberrant signals remains to be determined.

In addition to integrin-mediated cell adhesion, a number of other extracellular stimuli are known to induce activation and tyrosine phosphorylation of FAK (Schlaepfer *et al.*, 1999; Schaller, 2001). Interestingly, several of these stimuli, such as treatment of cells with prolactin, also induce SOCS protein expression (Naka *et al.*, 1999; Yasukawa *et al.*, 2000; Krebs and Hilton, 2001). FAK may therefore be a target for regulation by SOCS proteins in other signaling pathways, as well. Our preliminary studies indicate that signaling by the FAK-family member Pyk2 is also suppressed by SOCS proteins (data not shown). Pyk2 becomes activated by a number of cytokines that are known to regulate SOCS proteins, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Schlaepfer *et al.*, 1999; Avraham *et al.*, 2000). Of interest, Pyk2 has been suggested to be an important mediator of IFN-γ signaling (Takaoka *et al.*, 1999), and to mediate TNF-α-induced apoptosis (Avdi *et al.*, 2001). In this context, the analysis of mice lacking SOCS-1 has revealed that SOCS-1 is critical in the negative regulation of IFN-γ and in suppressing TNF-α-induced cell death in fibroblasts (Metcalf, 1999; Morita *et al.*, 2000). Additional future research is needed to clarify the physiological significance of FAK family proteins and their interactions with SOCS proteins in various signaling pathways.

Materials and methods

Cell culture and treatment

COS-7 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM/l glutamine, 100 U/ml penicillin and streptomycin. For cell adhesion experiments, COS-7 cells were serum-starved for 24 h. The cells were detached, maintained in suspension in DMEM/0.5% bovine serum albumin (BSA) at 37°C for 30 min, and then either kept in suspension for 30 min, or replated on FN-coated dishes (10 μg/ml) for the indicated time periods. When studying SOCS-3 expression (Figure 1), NIH 3T3 cells were serum-starved for 24 h in DMEM containing 1% FBS. The cells were detached, stimulated or not with 50 ng/ml hPDGF-BB (Roche Molecular Biochemicals), and either kept in suspension or replated on FN-coated dishes for 1 h. For ubiquitination experiments, cells were pretreated with 50 μM pervanadate for 60 min before analysis. Where indicated, the proteasome inhibitors MG132 or β-lactacystin (Sigma) at 25 μM were added to the cells 4 h before pervanadate treatment and maintained throughout the experiment.

Plasmids and transient transfections

Hemagglutinin (HA) epitope-tagged wild-type FAK and its autophosphorylation site mutant FAK-Y397F in the pcDNA3 vector were obtained from Dr David Schlaepfer. Myc-tagged wild-type SOCS-1, SOCS-3, CIS-1 and their single SH2- and KIR-domain mutants were kind gifts from Dr Akihiko Yoshimura. The SOCS-1 SH2-KIR-double mutant was generated by ligation of a 424 bp *Xcm*I–*Xba*I fragment of SOCS-1-R105K, which contained the R105K mutation in the SH2 domain, to *Xcm*I–*Xba*I-digested SOCS-1-F59EdN. The SOCS-3 SH2-KIR-double mutant was generated by creating a F25A mutation in the KIR domain of the SOCS-3-R71E construct by using QuikChange Site-Directed Mutagenesis Kit (Stratagene). HA-JAK2 was obtained from Dr Olli Silvennoinen, GST–Erk1 from Dr Gen-Sheng Feng, HA-FRNK from Dr Jun-Lin Guan, and HA-ubiquitin from Dr Dirk Bohmann. The plasmid pCMV-β-Gal coding for β-galactosidase was used in all migration assays to monitor the transfection efficiency. Transient transfection experiments were carried out in serum-free medium with the indicated amounts of plasmids by using Lipofectamine (Invitrogen).

Immunoprecipitations and immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a modified radioimmunoprecipitation assay (RIPA) lysis buffer [20 mM HEPES pH 7.3, 1% NP-40, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM PMSF and Protease Inhibitor Cocktail (Roche)]. Clarified cell extracts were immunoprecipitated with the indicated antibodies followed by SDS–PAGE analysis and immunoblotting coupled with enhanced chemiluminescence detection (ECL). For re-immunoprecipitation experiments, FAK immunecomplexes were eluted by boiling in 2% SDS, 150 mM NaCl for 3 min, and diluted to a final concentration of 0.1% SDS using lysis buffer and re-immunoprecipitated with the anti-HA antibody. The following antibodies were used in this study: anti-SOCS-3 (Immuno-Biological Laboratories, Japan), anti-pTyr (Transduction Laboratories), anti-FAK-pY397 (Biosource International), anti-FAK (N17, C-20), anti-Myc, anti-GST, anti-HA (all from Santa Cruz Biotechnology), anti-ubiquitin (Zymed), anti-phospho-MAPK (Cell Signaling Technology), and anti-Tubulin (Calbiochem).

SH2 domain binding assay

In vitro association experiments were performed with the GST fusion proteins of SOCS-3 covering the SH2-domain (amino acids V34–L182) with or without an R71E point mutation. The constructs were generated by PCR and subcloned to pGEX-4T-1 (Amersham). The fusion proteins were expressed in *Escherichia coli* and purified as described previously (Vuori *et al.*, 1996). Four hundred micrograms of precleared cell lysate was incubated for 2 h at 4°C with 5 µg of GST alone or of the GST fusion proteins which had been immobilized on glutathione–Sepharose beads. The beads were collected and washed extensively with RIPA lysis buffer and the bound proteins were released by boiling in sample buffer and then subjected to SDS–PAGE followed by immunoblot analysis.

Far-western blotting

Oligonucleotides (5′-CTCGGATCCATGGCAGCTTATCTTG-3′ and 5′-GTTCTCGAGTGTCTTCCTCATCG-3′) were used to amplify sequences coding for amino acids 1–406 of murine wild-type FAK and FAK-Y397F to generate GST–FAK-NT (WT) and GST–FAK-NT (Y397F) constructs in pGEX-4T-1, respectively. The constructs were transformed into the TKX1 strain (Stratagene), which harbors a plasmid-encoded, inducible tyrosine kinase gene. The induction of protein expression was carried out according to the manufacturer's protocols. Glutathione Sepharose-purified FAK-NT (WT) and FAK-NT (Y397F) were run on SDS–PAGE and transferred onto PVDF membrane. The membranes were blocked with binding buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM DTT and 0.5% BSA) for 2 h. The membranes were probed for 4 h at room temperature in the binding buffer with 1 µg/ml EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce)-labeled GST–SH2 fusion proteins of either Src (Vuori *et al.*, 1996), SOCS-3 or SOCS-3-R71E. The blots were washed four times with binding buffer and developed with peroxidase-conjugated ExtrAvidin (Sigma) and ECL.

RT–PCR

NIH 3T3 cells were washed twice with ice-cold PBS, and lysed in Trizol (Invitrogen). Total RNA was isolated as per manufacturer's instructions and subjected to reverse transcription using Superscript II Reverse Transcriptase (Invitrogen). Primers used for SOCS-1 amplification were 5′-ACACTCACTTCCGCACCTTC-3′ and 5′-GAAGCCATCTTCACG-CTGAG-3′. Primers used for GAPDH amplification were 5′-TTC-ATTGACCTCAACTACATGG-3′ and 5′-GTGGCAGTGATGGCAT-GGAC-3′. PCRs were carried out with Expand High Fidelity enzyme (Roche) (30 s denaturing at 94°C, 30 s annealing at 55°C and 30 s extension at 72°C). The linearity of the reactions was verified by removing sample aliquots after 25, 30 and 35 cycles.

In vitro kinase assay

HA-tagged FAK was immunoprecipitated from transiently transfected COS-7 cells and the immunocomplexes were washed extensively in kinase assay buffer (20 mM HEPES pH 7.4, 10 mM $MnCl_2$, 100 µM vanadate, 0.05% Triton X-100), and then incubated in kinase assay buffer containing 0.25 µCi/µl of [γ-^{32}P]ATP (6000 Ci/mmol; DuPont-NEN) for 15 min at 30°C. In some experiments, 20 µg of poly(Glu:Tyr) (4:1; Sigma) was added to FAK immunoprecipitates as an exogenous substrate, and kinase reactions were initiated by ATP addition (final concentrations: 3 µM ATP, 3 µCi/sample of [γ-^{32}P]ATP). The reactions were stopped by addition of an equal volume of 2× SDS–PAGE sample buffer, and analyzed by SDS–PAGE followed by autoradiography.

Pulse–chase experiments

Twenty-five micromolar β-lactacystin was added or not to transiently transfected COS-7 cells 1 h prior to cell manipulations where indicated and maintained throughout the experiment. The cells were then starved in methionine- and cysteine-free DMEM (Gibco-BRL) for 15 min, pulsed with medium containing 100 µCi/ml [^{35}S]methionine and [^{35}S]cysteine (Promix; Amersham) for 30 min, and chased for 1, 2 or 4 h. Equivalent amounts of protein extracts, as determined by the Bradford assay, were immunoprecipitated with anti-HA antibody to recover FAK. The immunoprecipitates were separated on SDS–PAGE, and the gels were treated with Amplify (Amersham), dried and visualized by autoradiography.

In vitro ubiquitination assay

HA-tagged wild-type FAK and FAK-Y397F from either transfected COS-7 cells, or HA-tagged wild-type FAK from a coupled *in vitro* transcription/translation reaction (TnT, Promega) were immunoprecipitated with anti-HA antibody and purified by using protein A-Sepharose beads. After washing extensively with lysis buffer, the beads were resuspended in 50 µl reaction buffer containing 20 mM HEPES pH 7.3, 10 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 25 mM MG132, 5 µg/ml ubiquitin, 20 µg/ml ubiquitin aldehyde and 100 µg of lysates from COS-7 cells transfected with either wild-type SOCS-1, SOCS-1ΔSB, wild-type SOCS-3, SOCS-3ΔSB or a control vector. Five microliters of crude rabbit reticulocyte lysates were added to each reaction. To deplete endogenous ATP, 1 mg/ml of hexokinase and 20 mM 2-deoxyglucose were also added. The reaction mixture was incubated for 1.5 h at 30°C, the Sepharose beads were washed extensively with lysis buffer containing 25 mM MG132, protease and phosphatase inhibitors, resuspended in SDS loading buffer, separated on SDS–PAGE and transferred onto PVDF membrane. The blots were probed with anti-ubiquitin antibody.

Migration assays

Cell migration assays were performed using 24-well Transwell chambers (Costar). In haptotactic migration assays, the underside of the membrane was coated with 10 µg/ml FN in PBS for 2 h at 37°C and blocked with 0.5% BSA in PBS. In chemotactic migration assays, both sides of the membrane were coated with FN, and 10 ng/ml hPDGF-BB was added to the bottom chamber. Serum-starved cells were detached with Hanks' balanced salt solution containing 5 mM EDTA, 25 mM HEPES, pH 7.2, and 0.01% trypsin, washed twice with migration buffer (fibroblast basal medium with 0.5% BSA), and resuspended in the same buffer at 10^6 cells/ml. Cells (1×10^5) were added to the upper migration chamber and allowed to migrate for 3 h at 37°C, the non-migratory cells on the upper membrane surface were removed with a cotton tip, and the migratory cells attached to the lower membrane surface were fixed and analyzed for β-galactosidase activity using X-gal as a substrate. Cell migration values were determined by counting stained cells (cells/field using a 40× objective). Each determination represents the average of three individual wells, and error bars represent the standard deviation. Background values of cell migration to 0.5% BSA were <1% of values obtained with FN. Data presented are representative of three independent experiments.

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